University of Alberta

IN VITRO CHOLESTEROL AND BILE ACID BINDING CAPACITY OF SAPONINS EXTRACTED FROM FENUGREEK (*Trigonella foenum-graecum* L.) GROWN IN ALBERTA, CANADA

BY

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Dedication

This thesis is dedicated with love and respect to my parents, Mr Kanti Bhaumick and Mrs Lila Bhaumick in recognition of their effort in raising me and teaching me to become an honest human being.

ABSTRACT

Saponins are glycosidic compounds consisting of sapogenin moiety with one or more sugar molecules. The hypocholesterolaemic activity of saponins is assumed due to the formation of complexes with dietary cholesterol or with bile acid. The present study was undertaken a) to extract saponins from seeds of a Canadian-grown fenugreek (*Trigonells foenum-graecum* L. Var. Amber) and b) to examine *in vitro* cholesterol and bile acid-binding capacities of the saponin-rich fractions of fenugreek seed extract. Saponin was extracted with 80% ethanol followed by purification through C-18 Sep-Pak column. The crude and purified extracts contained 25.42 μg/mg and 109.1 μg /mg of diosgenin (major sapogenin in fenugreek seeds) respectively.

The crude extract displayed a greater ability to bind cholesterol than that of the purified extract (41.8% vs 35.4%; $p < 0.001$). *In vitro* taurocholic acid binding with different concentrations of purified extract was found to be purified extract concentration-related. These results suggest that the crude extract of fenugreek seeds, grown in Alberta, Canada, have the ability to bind both cholesterol and bile acid, and hence the potential to modify cholesterol metabolism.

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TABLE OF CONTENTS

Chapter 1. Introduction..... 1

1.1. Fenugreek (*Trigonella foenum-graecum* L.)..... 2

1.1.1. Cultivation..... 3

1.1.2. Traditional uses..... 4

1.1.3. Chemistry..... 4
1.1.3.1. Galactomannan..... 5
1.1.3.2. 4-Hydroxyisolucine..... 8
1.1.3.3. Saponins..... 9

1.1.4. Health benefits..... 12
1.1.4.1. Dietary prevention of Diabetes Mellitus (DM) 12
1.1.4.2. Antidiabetic activity of fenugreek..... 13
1.1.4.3. Hypocholesterolaemic activity of fenugreek 16

1.2. Hypothesis and Objectives..... 21

1.3. References 23

Chapter 2. Extraction and purification of saponin from Canadian fenugreek seeds..... 36

2.1. Introduction 36

2.2. Materials and Methods..... 40

2.2.1. Plant Materials..... 40

2.2.2. Chemicals..... 40

2.2.3. Lipid extraction..... 41
2.2.3.1. Defatting with petroleum ether and hexane 41
2.2.3.2. Super critical fluid extraction..... 41

2.2.4. Extraction of saponins..... 42
2.2.4.1. Procedure 1..... 42
2.2.4.2. Procedure 2..... 43
2.2.4.3. Procedure 3..... 43
2.2.4.4. Procedure 4..... 43

2.2.5. Purification..... 44
2.2.5.1. Purification procedure 1..... 44
2.2.5.2. Purification procedure 2..... 45
2.2.5.3. Purification procedure 3..... 46

2.2.6. Identification of fenugreek saponin..... 46
2.2.7. Statistical analysis..... 47

2.3. Results and Discussion 48

2.2.1. Lipid extraction..... 48
2.2.2. Extraction of saponins..... 49
2.2.3. Purification of saponins..... 51

2.4. References 54

Chapter 3. Analysis of crude extract and purified extract..... 60

3.1. Introduction 60

3.2. Materials and Methods..... 66

3.2.1. Determination of soluble and insoluble dietary fiber in crude extract and purified extract 66

3.2.1.1. Chemicals..... 66
3.2.1.2. Procedure..... 67

3.2.2. Determination of sapogenins in crude extract and purified extract 68
3.2.3. Determination of sugars in crude extract and purified extract..... 70
3.2.4. Statistical analysis..... 72

3.3. Result and Discussion 72

3.3.1. Determination of the fiber in crude extract..... 72
3.3.2. Determination of fiber in purified extract..... 73

3.3.3. Determination of sapogenins in crude extract and purified extract 73
3.3.4. Determination of sugars in crude extract and purified extract 77

3.4. References 82

Chapter 4. Cholesterol and Bile acid binding capacity of crude extract and purified extract 86

4.1. Introduction 86

4.2. Materials and Methods 88

4.2.1. *In vitro* cholesterol binding..... 88
4.2.1.1. Preparation of standard curve..... 88
4.2.1.2. Cholesterol binding with crude extract and purified extract..... 89

4.2.2. *In vitro* bile acid binding..... 90
4.2.3. Effect of purified extract on bile acid binding..... 91
4.2.4. Statistical analysis..... 92

4.3. Result and Discussion 92

4.3.1. Cholesterol binding..... 92
4.3.2. Bile acid binding 94

4.4. References 98

Chapter 5. General Discussion 100

5.1. Conclusion..... 110

5.2. References 112

LIST OF TABLES

Table 1.	Chemical structure and functionality of Guar, LBG, Tara and Fenugreek	6
Table 2.1	Effect of defatting of lipid and lipid like compounds	48
Table 2.2	Effect of various extraction procedures on extraction of saponin	50
Table 3.1	Estimation of soluble and insoluble dietary fiber in crude extract	73
Table 3.2	Estimation of sapogenins in crude extract and purified extract	76
Table 3.3	Purification of diosgenin (in percent) by the sep-pak method	77
Table 3.6	Estimation of sugars (%) present in crude extract and purified extract	79
Table 4.1	Significant food and beverage plants which contain saponins	86

LIST OF FIGURES

Figure 1.1	Structure of fenugreek galactomannan	7
Figure 1.2	Different isomers of 4-Hydroxyisolucine	8
Figure 1.3	Structure of fenugreek saponin	10
Figure 1.4	Transformation of fenugreek saponin in the digestive tract	11
Figure 2.1	Schematic representation of the extraction and purification process	53
Figure 3.1	Chemical structure of different sapogenins	60
Figure 3.2	Chemical structures of saponins	61
Figure 3.3	Chromatogram from GC analysis of fenugreek crude extract	75
Figure 3.4	Chromatogram from GC analysis of purified extract	75
Figure 3.5	GC Chromatogram showing carbohydrates present in crude extract	78
Figure 3.6	GC Chromatogram showing carbohydrates present in purified extract	78
Figure 3.7	Effect of duration of hydrolysis on total carbohydrate (%) extraction of crude extract and purified extract.	80
Figure 4.1	Standard curve for cholesterol	93
Figure 4.2	Effect of crude extract and purified extract on cholesterol binding.	94
Figure 4.3	The dose dependent binding of [14C] taurocholate by crude extract	95
Figure 4.4	The dose dependent binding of [14C] taurocholate by purified extract	95
Figure 4.6	Bile acid ([14C] sodium taurocholate) binding by purified extract	96

CHAPTER 1

Introduction

Legumes are plants belonging to the family Leguminosae. A large number of species of legumes (such as beans, lentils or chickpeas) is cultivated throughout the world, primarily for their seeds, which are harvested at maturity and marketed as dry products, generally referred to as pulses. The latter are rich in protein, starch, dietary fiber and inorganic elements (Geil and Anderson, 1994; Messina, 1999). In addition to these nutritional factors, pulses are known to contain a number of non-nutritive bioactive substances, such as polyphenols (Mazure, 1998), saponins (Oboh et al., 1998), lectins (Bond and Duc, 1993) and enzyme inhibitors (Leterme et al., 1992). The nutrients, dietary fiber and the non-nutritional bioactive components of legumes have useful functional properties, which in recent years have encountered a revival of interest from the consumers. Once known as the poor man's meat, legumes, such as beans are now presented as a staple food for vegetarians, and most of the health organizations encourage their frequent consumption (Messina and Burke, 1997). Legumes have low glycemic indices (Foster-Powell and Miller, 1995); they have been shown to have hypocholesterolaemic effects (Anderson et al., 1999); and they contain phytochemicals that may act as chemo preventive agents (Lippmann et al., 1998). However, it becomes increasingly clear that the health potentials of different legumes vary significantly. These differences may result from variations in nutritional and non-nutritional factors, in particular quantity and variety of dietary fiber, protein make-up, and the phytochemical and saponin contents. In effect, with the exception of

soybeans, few nutritional studies have been carried out in the area of legumes, and even fewer have distinguished between the individual characteristics of this heterogeneous family of food. Hence, investigation of less commonly consumed legumes may reveal new sources of functional foods, which will prevent disease and even act as therapeutic agents.

Fenugreek is an annual crop from the family Leguminosae. The seed of this plant, grown in South Asia, has been known to have health potential with the ability to lower blood glucose and cholesterol levels, and hence in the prevention and treatment of diabetes and coronary heart disease. It is only recently that fenugreek has become a speciality crop in the Western Prairies of Canada, primarily for its leaves to be used for animal feed as forage. There is very little information available on this Canada grown legume.

1.1. Fenugreek (*Trigonella foenum-graecum* L.)

Fenugreek, a legume grown in Asia and Middle East, has been used for centuries in folk medicine to heal ailments ranging from indigestion to baldness. The species name foenum-graecum means "Greek hay", while the genus name Trigonella means "triangle" referring to the triangular shape of its leaflets. There are many local names used for fenugreek. Thus, the name "Methi" is used in India and Pakistan, "Pazhitnik" in Russia and in Arabic countries it is known as Helbeh".

1.1.1. Cultivation

Fenugreek is an annual plant, yielding seeds in 3-4 months after sowing. The ideal growing conditions include temperature ranging from 8 to 27 C, annual precipitation of 41 to 152 cm and soil pH of 5.4 to 8.0. Fenugreek plants grow well in full sun, rich and well drained soil and cold temperature; water logged conditions slow down the growth. The leaves of the plant alternate and consist of three ovate leaflets. The plant stalk grows up to 120 cm tall. White flowers appear in early summer and then develop into long slender green pods. The best ripening of the pod occurs in warm and dry conditions. Prior to pod formation the young green tops of fenugreek may be cut as a vegetable. Each mature brown pod may contain as many as 20 small yellow to brownish yellow seeds, which are extracted from the pods by using a thresher. The hay is used for animal feed and the roots with nodules are left to enrich the soil with nitrogen. The seeds are about 0.3 to 0.4 cm long, rhomboidal with a deep furrow dividing each seed into two equal halves.

In recent years, fenugreek has been a specialty crop in western Canada. Its adaptation to the dry land conditions and annual nature makes it suitable for incorporation into short term crop rotations. At present, fenugreek is grown in Canada for its seed which is used as a condiment and flavoring artificial maple syrup. However, the acreage for this crop is small at present. Since fenugreek has high feed value and its seeds have steroidal compounds, Lethbridge Research Centre of Agriculture and Agri-Food Canada is now in the process of developing this for its use as a forage crop.

1.1.2. Traditional uses

Fenugreek is used for a variety of purposes. In North America, fenugreek seed extract is used as a flavoring ingredient of artificial maple syrup (Abdel-Nabey and Damir, 1990). It is also used as a tobacco flavoring ingredient, hydrolyzed vegetable protein flavor, perfume base and source of steroid sapogenin in drug manufacturing industries (Sorengarten, 1969; Leung and Foster, 1996). In Indian subcontinent, the ground seeds are used is spice mixtures and as a condiment. The leaves are commonly consumed as a vegetable (Patil et al., 1997). In Egypt, the seeds are either eaten raw sprouting or are used to make a confection following roasting and grinding (Al-Habori and Raman, 1998). Seeds are also added to maize and corn flower in bread making.

1.1.3. Chemistry

The nutrient composition of fenugreek seed consists of approximately 30% protein, 26% starch, 13% natural detergent fiber, 4% gum, 6% lipids and 11% ash. It is also rich in calcium, iron, β-carotene and other vitamins (Gupta et al., 1998a; 1998b). In comparison to other legumes, fenugreek seeds contain higher proportions of minerals including Ca, P, Fe, Zn and Mn. Trigonelline is an important alkaloid component of the seeds (Shani et al., 1974), which also contain some aromatic constituents such as n-alkenes, sesquiterpenes and nonalactone. They have been

found also to be rich in saponins, including diosgenin, gitogenin and tigogenin (Dawidar et al., 1973).

There are three potential bioactive components that have been suggested to be associated with fenugreek seeds. These include galactomannans, 4-hydroxyisolucin and saponins.

1.1.3.1. Galactomannan

Galactomannans are a group of hydrocolloids (gums) that are widely used in the food industry as thickeners, binders, stabilizers, emulsifiers, and suspending or gelling agents, because they are functional, available, and low in cost (Wistler, 1993). Their functional and physical properties (including solubility, gelling behavior, and viscosity) are related to the molecular structure, sugar composition, degree and distribution of branching, and polymerization (Stephen and Churms, 1995); these properties are the focus of several reviews (Dea et al., 1975; Whistler et al., 1997). The structural details of select gums are given in Table 1.1

Table 1.1. Chemical Structure and functionality of Guar, Locust bean gum (LBG), Tara and Fenugreek.

Gum	Structure		M/G ratio	Branch distribution	Mol. weight	Viscosity (cP) (1% solutions)	Functionality	Synergy	Source
	Backbone	Side chains							
Guar	(1→4)-β -D-Man*p* branched every other Man*p* at O-6	-(6→1)-α -D-Gal*p*	1.8:1	nonuniform	220 000	4600	high viscosity and pseudoplastic at low concentrations; formulation aid, stabilizer, thickener, emulsifier, firming agent	xanthan gum, starches, cellulose, agar, κ-carrageenan	*Cyamopsis tetragonolobus*
LBG	(1→4)-β -D-Man*p* branched in clusters in Man*p* at O-6	-(6→1)-α -D-Gal*p*	3.9:1	clustered	310 000	2400-2300	thixotropic, binder, lubricator, and stabilizer; provides heat-shock resistance in ice cream products; speeds coagulation of cheeses	agar, κ-carrageenan, xanthan	*Ceratonia siliqua*
Tara	(1→4)-β -D-Man*p* branched in clusters in Man*p* at O-6	-(6→1)-α -D-Gal*p*	2:1				higher viscosity than guar or LBG, water holding capacity, protective colloidal properties, interfacial tension activity	carrageenan and agar	*Cesalpina spinosum*
Fenugreek	(1→4)-β -D-Man*p* branched in clusters in Man*p* at O-6	-(6→1)-α -D-Gal*p*	1:1	uniform,		Mucila-ginous solutions	lower viscosity than guar and LBG at the same concentration and molecular weight; reduces surface tension		*Trigonella foenumgraecum*

Note: D-Man*p* = D-mannopyranosyl; D-Gal*p* = D-galactopyranosyl; M/G ratio = Mannose and Galactose ratio.

The seeds of many leguminous plants have mucilaginous endosperm (Meier and Reid, 1977). The endosperms of many leguminous seeds contain galactomannan (Reid and Meier, 1970). The major source of stored carbohydrate reserves in the dry fenugreek seed is the galactomannan contained within the endosperm cell walls (Reid and Bewley, 1997). Galactomannans, which belong to a family of seed gums and represent polymers of galactose and mannose (Buckeridge et al., 1995), consisting of a backbone of (1→4)-linked β-D-mannopyranosyl units with chains of (1→6)-α -D-galactopyranosyl groups (Fig. 1.1). Guar gum, locust bean gum (LBG), tara gum, and fenugreek gum are galactomannans that have different mannose/galactose (M/G)

ratios and distributions of galactopyranosyl units along the mannan chains. The latter has galactose and mannose residues in the ratios of 1:1 or in few cases of 1:2 (Garti et al., 1997). It has been found that by increasing viscocity of the digesta in the gut, these fibers delay the absorption of carbohydrates, thereby affecting postprandial plasma glucose excursion (Evans et al., 1992). Further, they have also been found to exert a hypoglycemic effect by increasing the short chain fatty acid mediated production of Glucagon-like peptide – 1 (GLP-1) in the intestine (Groop et al., 1993). Antidiabetic effect of GLP-1 including the ability to stimulate insulin secretion, inhibit glucagon production, and delay gastric emptying has been documented in a number of studies (Massimino et al., 1998). These fibers have also been shown to be effective in lowering lipids and systolic blood pressure (Vuksan et al., 2001).

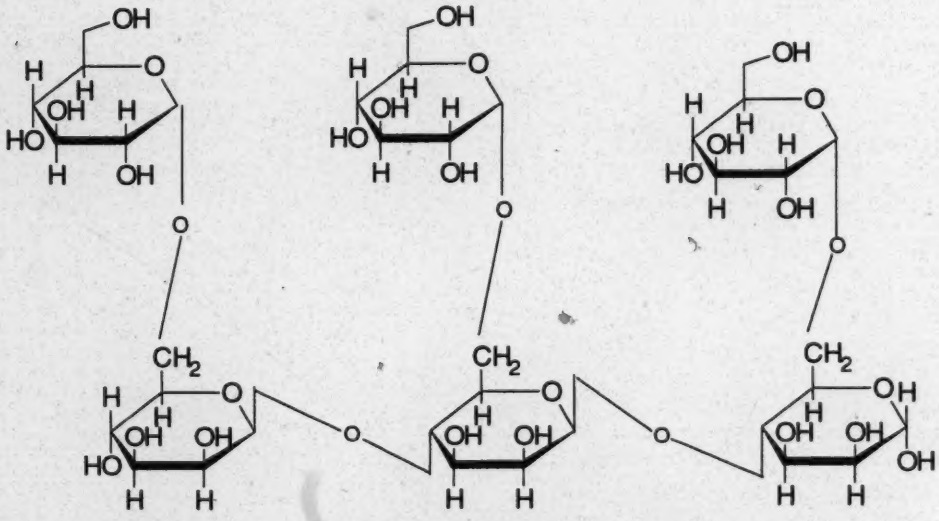

Figure 1.1: Structure of fenugreek galactomannan

1.1.3.2. 4-Hydroxyisoleucine

4-Hydroxyisoleucine (4-OH-Ile), a polar non-protein amino acid, structurally related to branch chain amino acids, is not present in mammalian tissues. It is a molecule with 3 chiral centers, which has been found only in very specific plants, especially *Trigonella* species (Sauvaire et al., 1998). In fenugreek seeds, it accounts for approximately 80% of the total content of the free amino acids (Gupta et al., 1999). The amino acid is present in the seeds as two diastereoisomers. The major one has been found to have 2S, 3R, 4S configuration and represents up to 90% of total 4-OH-Ile content of the seeds (Fig. 1.2). The minor one possesses 2R, 3R, 4S configuration. The major isomer has been found to be a strong insulinotropic compound in both *in vitro* and *in vivo* studies (Sauvaire et al., 1998; Broca et al., 1999). The effect of 4-OH-Ile was shown to be strictly glucose dependent and occurred without affecting the functions of other pancreatic cells (Sauvaire et al., 199

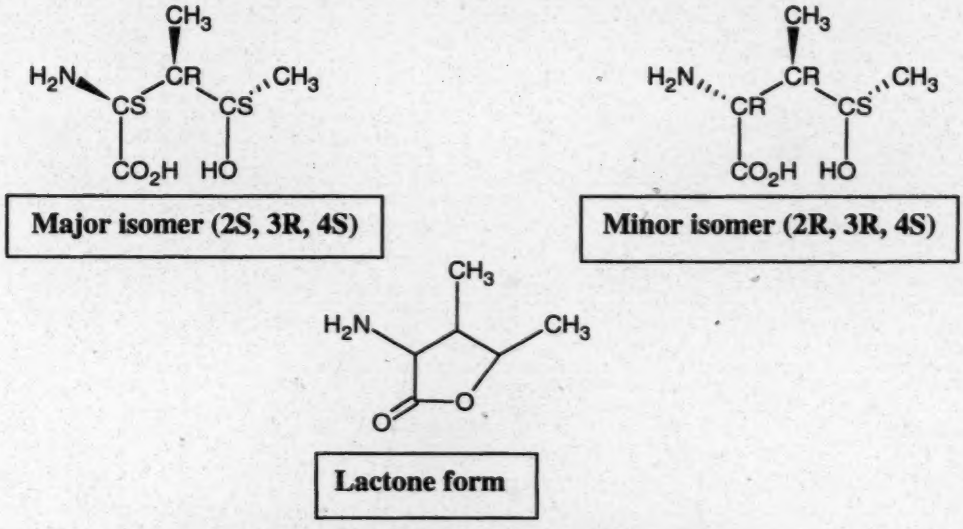

Figure 1.2: Different isomers of 4-Hydroxyisolucine

Another important feature of 4-OH-Ile molecule is its linear form. It was found that cyclization of the structure to the lactone form resulted in significant alteration of its chemistry (Fig. 1.2). This form of 4-OH-Ile, however, has been found to be biologically inactive (Broca et al., 2000). The linear configuration is thus required to produce the desired 'antidiabetic effect'. Full branching along the carbon skeleton is another important property of the major isomer (Broca et al., 2000). It was found that removal of the methyl group from asymmetric carbon γ or β significantly reduced the effectiveness of the molecule as a stimulator of insulin release. Although some other structurally related amino acids have exhibited insulinotropic properties in the presence of stimulating glucose concentration, none is as effective as 4-OH-Ile. The stimulating threshold concentration of 4-OH-Ile is at least 10 fold lower than the concentrations required of these other branched-chain amino acids. The structure-activity relationships between 4-OH-Ile and closely related substances point to the fact that the full branching along the carbon skeleton, carbon γ hydroxylation and carbon α S configuration are the three main characteristics that determine the insulinotropic effect of 4-OH-Ile.

1.1.3.3. Saponins

Saponins are a diverse group of glycoside compounds, which are composed of a lipid-soluble aglycone consisting of a sterol or more commonly a triterpenoid structure attached to a water-soluble sugar molecule (Fig. 1.3).

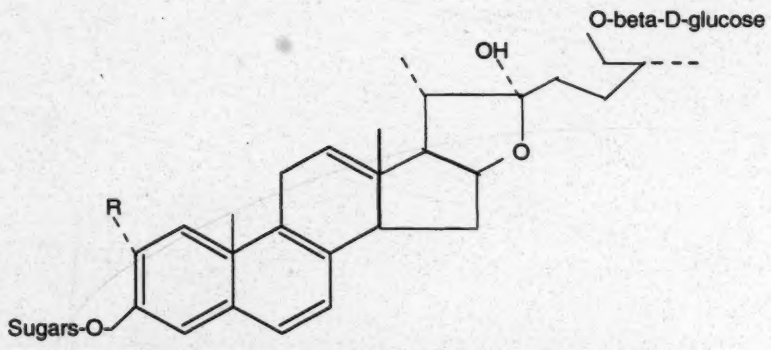

Figure 1.3: Structure of fenugreek saponin

Fenugreek seeds are rich in steroidal saponins, which compose 5-6% of the seeds (Sharma, 1986; Sauvaire et al, 1996). The extracted saponins from fenugreek seeds were found to be exclusively the furostanol form (Petit et al, 1995). Approximately 57% of the saponins are hydrolyzed into sapogenins in the digestive tract (Sauvaire et al, 1991) (Fig. 1.4).

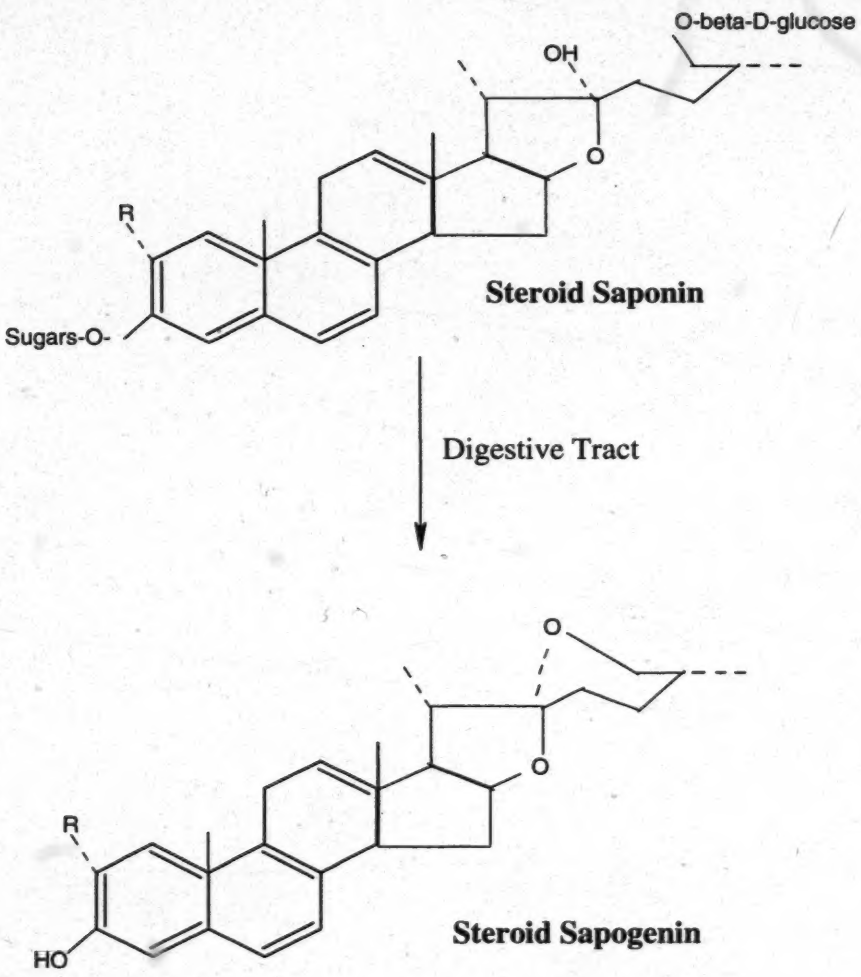

Figure 1.4: Transformation of fenugreek saponin in the digestive tract

Saponins have a characteristic bitter taste (Bink and Peri, 1980). They are poorly absorbed and hence their effects are attributable to their hydrophobic/hydrophilic asymmetry and consequently their capacity to reduce interfacial tension. Erythrocytes are thus disrupted in the saponin solution due to an interaction with cholesterol in the erythrocyte membrane (Birk and Peri, 1980).

Saponins form insoluble complexes with 3-β-hydroxy steroids and are known to interact with, and form large mixed micelles with bile acids and cholesterol.

1.1.4. Health Benefits

1.1.4.1. Dietary Prevention of Diabetes Mellitus (DM)

The prevalence of DM is rapidly increasing across the world. Diet is suspected to play a decisive role in the increasing burden of this chronic disease. In recent years, interest in administering fiber-rich diets to diabetic patients or those with glucose intolerance has been increasing since it has been proven that it improves glycemic control and, at the same time lowering insulin requirements (Anderson and Ward, 1979; Jenkins and Jenkins, 1985; Weinstock and Levine, 1988).

Soluble fiber (such as guar gum, beta-glucan and galactomannan) is more effective in improving glycemic control than the insoluble fiber (such as cellulose and some hemicelluloses). The mechanisms whereby dietary fiber is capable of improving the homeostasis of glucose in diabetic individuals are thought to be mediated by delaying gastric emptying, trapping of sugars in the matrix of the fiber and thus affecting their absorption followed by reduction of glucose levels and modifying insulin secretion (Weinstock and Levine, 1988).

Besides improving glycemic control, another advantage of administering fiber to the diabetic patient is the prevention of late complications and especially cardiovascular disease.

Although the role played by fiber in the regulation of hyper-triglyceridaemia has not yet been understood, some studies have reported an association between the ingestion of a fiber-rich diet and the reduction of the levels of triglycerides in hyper-trigliceridaemic patients (Anderson et al., 1980).

1.1.4.2. Antidiabetic activity of fenugreek

According to many studies (Ribes et al., 1986; Madar et al., 1988; Sharma and Raghuram, 1990; Sharma et al., 1990; Khosla et al., 1995), fenugreek appears to be a potential hypoglycemic food commodity in both healthy and diabetic subjects. It has thus been shown to reduce the post-prandial glucose response as evidenced by reduction in the area under the glucose curve by 35-42% (Sharma, 1986). This was true for whole fenugreek seed, defatted seed, or its gum isolate. Similar results were observed in patients with non-insulin dependent diabetes mellitus (NIDDM) when 15 g of ground fenugreek seed was added to a meal tolerance test (Madar et al., 1988). These results were further confirmed in longer metabolic studies of 10 day to 2 month in both NIDDM and in patients with insulin dependent diabetes mellitus (IDDM) consuming 100 g/d of defatted fenugreek or fenugreek extract (Sharma and Raghuram, 1990; Sharma et al., 1990; Gupta et al., 2001). While the beneficial effect of fenugreek with regards to glycemic control is well documented, the mechanisms through which the legume exerts this effect are still not well understood. There have been few animal studies carried out to examine the possible underlying mechanism(s)

of the hypoglycemic action of fenugreek. Using streptozotocin (STZ) induced diabetic rats, administration of ground fenugreek seeds has thus shown to have a profoundly decreased post prandial glucose tolerance curves in association with a significantly reduced rate of gastric emptying (Madar, 1984). Inhibited glucose treatment was also observed *in vitro* using inverted sections of rat intestines incubated with fenugreek (Madar, 1984).

The highly viscous gel fraction of fenugreek, rich in galactomannans, has also been reported to be an effective hypoglycemic agent (Madar and Shomer, 1990). It has also been shown to inhibit glucose absorption from perfused loops of small intestine (Evans et al., 1992). It appears that the soluble dietary fiber in fenugreek, acting in the intestinal tract, is largely responsible for its strong hypoglycemic action; it is biologically active in both ground whole seeds and as a purified isolate. This is also supported by the fact that in contrast to whole seed or its purified isolate, degummed seeds have little effect on glycemic response (Sharma, 1986).

One study examined the effect of oral administration of fenugreek whole seed powder on glycolytic, gluconeogenic and NADP-linked lipogenic enzymes in the liver and kidney tissues of alloxan-induced diabetic Wister rats. This study showed that the altered enzyme activities were significantly restored to control values in both the liver and kidney after *Trigonella* seed powder treatment (Raju et al., 2001).

The hypoglycemic effect of the fenugreek may also be attributed to the 4-hydoxyisolucine present in the fenugreek seeds. Hillaire-Buys et al. (1993) were the first to report the presence of the amino acid in the seeds of fenugreek and that the

amino acid had insulin stimulating property. Using isolated pancreas perfused with glucose, 4-hydroxyisolucine evoked a biphasic insulin response at a concentration of 200 µmol/l (Petit et al., 1995; Sauvaire et al., 1996). 4-Hydroxyisolucine, which represents up to 80% of the free amino acids in fenugreek seeds (Sauvaire et al., 1984), was found to stimulate insulin secretion only in the presence of intermediate to high glucose concentrations and to be effective in a much lower concentration range than its structural amino acid congeners' leucine and isoleucine. 4-Hydroxyisolucine has been shown to increase glucose-induced insulin release in human and rat pancreatic islet cells (Sauvaire et al., 1998). This amino acid appeared to act only on pancreatic beta cells, since the levels of somatostatin and glucagon were not altered.

More recently, Broca et al. (1999, 2000) have demonstrated that 4-OH-Ile, has both insulinotropic and anti-diabetic properties. Intravenous and oral glucose tolerance tests were thus carried out in healthy dogs and rats. The addition of the 4-OH-Ile improved glucose tolerance in these animals. Furthermore, single administration of the 4-OH-Ile to IDDM rats partially restored a glucose induced insulin response. A sub-chronic administration of 4-OH-Ile reduced basal hypoglycemia decreased basal insulinemia and treated to improve overall glucose tolerance. An *in-vitro* study carried by the same investigators indicated that the amino acid directly caused pancreatic beta-cell stimulation. This stimulation was seen in the micro-molar change, but only the linear isoform of the amino acid (Broca et al., 2000). It appears that the protein fraction of fenugreek contains biologically active components that may be useful in both prevention and treatment of diabetes.

1.1.4.3. Hypocholesterolemic activity of fenugreek

Cardiovascular disease (CVD) is the number one killer disease in Canada and a major cause of hospitalization for men and women (excluding childbirth) (Heart and Stroke Foundation of Canada, 2000; Reeder et al., 1997). Among individuals of all ages, 36% of all deaths are attributed to CVD (Heart and Stroke Foundation, Canada, 2000). Coronary heart disease (CHD) remains the leading cause of death and disability in our society.

According to the Framingham Heart Study high blood cholesterol is a risk factor for coronary heart disease (CHD). Results of this study showed that higher the cholesterol level, greater the CHD risk. A direct link between high blood cholesterol and CHD has been confirmed by the Lipid Research Clinics-Coronary Primary Prevention Trial (1984) which showed that lowering total and LDL cholesterol levels significantly reduces CHD. Serum cholesterol is one of the main risk factors for coronary heart disease, and in Western countries the prevalence of elevated cholesterol levels is high (LaRosa et al., 1990). The cornerstone of therapy for CHD is its prevention through the modification of risk factors.

A number of studies have shown that saponins from different sources lower serum cholesterol levels in a variety of animals including human subjects (Southon et al. 1988; Harwood et al., 1993; Potter et al., 1993; Matsuura, 2001; Al-Habori and Raman, 1998). Saponin-rich foods (such as soybean, alfalfa, chickpeas etc), through

interacting with bile acids form mixed micelles, increased the excretion of bile acids (Oakenfull, 1986; Oakenfull and Sidhu, 1990). To compensate the requirement of bile acid; more bile acids are produced from cholesterol in the liver; causing the decrease of serum cholesterol level.

Decreased intestinal cholesterol absorption caused by some saponins, however, was seen to be without interference with the entero-hepatic bile acid recirculation (Hardwood et al., 1993). Saponin reduced serum LDL-cholesterol selectively in rats, gerbils and human subjects (Potter et al., 1993; Harris et al., 1997; Matsuura, 2001). Morehouse et al. (1999) found that the mechanism of action of saponins was luminal but did not involve stoichiometric complexation with cholesterol. Other suggested mechanism of action of saponins includes delaying the intestinal absorption of dietary fat by inhibiting pancreatic lipase activity (Han et al., 2000).

Fenugreek seeds have shown hypocholesterolemic effect in experimental animals (Singhal et al., 1982; Sharma, 1984, 1986; Stark and Madar, 1993; Valette et al., 1984; Khosla et al., 1995). Diet-induced elevation of cholesterol in rats was prevented by adding 150, 300 or 600 g fenugreek seeds/kg. Fenugreek seeds were demonstrated to have a grater effect on exogenous cholesterol (when given with a hypercholesterolaemia inducing diet containing 1% cholesterol) than on endogenous cholesterol (fenugreek given with a cholesterol free stock diet) (Sharma, 1984).

Diets enriched with fenugreek increased both fecal weight and excretions of bile acid and cholesterol. Bhat et al. (1985) reported that the administration of

fenugreek to rats increased total bile output. This was attributed to increased conversion of cholesterol to bile acids by the liver.

Studies carried out in human subjects fed 100 g of defatted fenugreek seeds powder for a short (10 days) period, resulted in decreased levels of serum total cholesterol, LDL and VLDL cholesterol and triglyceride levels without alteration of the HDL cholesterol levels (Sharma & Raghuram, 1990).

Defatted fenugreek seeds (100g) incorporated in the experimental diet of hyperlipidaemic non-diabetic human subjects significantly reduced serum total cholesterol, LDL and VLDL cholesterol and triglyceride levels with no observed change in HDL cholesterol level (Sharma et al., 1991). As a result, there was a significant increase in the ratio of HDL cholesterol to total cholesterol and HDL to LDL and VLDL cholesterol, which have been shown to be reliable risk assessment factors of CHD (Kannel, 1983).

In NIDDM patients, ingestion of an experimental diet containing 25 g fenugreek seed powder for 24 weeks resulted in a significant reduction of total cholesterol, LDL and VLDL cholesterol and triglyceride levels (Sharma et al., 1996). The reduction in LDL and VLDL concentrations are causing the decrease in total serum cholesterol. Triglyceride levels also showed a similar change. The HDL cholesterol showed a significant rise. The overall results are in agreement with earlier observations made in diabetic patients (Sharma, 1986, 1986a; Sharma et al., 1990). All the lipid parameters improved rapidly during the initial 8 weeks after the incorporation of fenugreek with a slower change thereafter (Sharma et al., 1991).

When germinated fenugreek seeds were added to the diet of hypercholesterolemic subjects (12.5 or 18.0 g/day) for 30 days, total cholesterol levels and LDL cholesterol levels were significantly reduced (Sowmya and Rakyalashmi, 1999). In type-2 diabetics, 1 g/day of a hydroalcoholic extract added to the daily diet for a 2-month period significantly decreased serum triglycerides and increased HDL cholesterol (Gupta et al., 2001). These results indicate a potential beneficial effect of fenugreek seeds in the lipid profile of diabetic as well as NIDDM subjects. It also appears that fenugreek has the ability to selectively reduce the LDL and VLDL fractions of total cholesterol, which could be considered as beneficial in preventing atherosclerosis.

Among the fenugreek fractions, lipid extract and 0.12% trigonelline had no hypocholesterolemic effect (Valette et al., 1984) while the defatted fractions, gum isolate and the crude saponins, fed to normal and diabetic rats at equivalent amounts present in a diet containing 30% fenugreek seeds, showed hypocholesterolemic activity without any significant effect on the triglyceride level (Sharma, 1986).

Ribes et al. (1987) showed that although sub fraction 'a' (79.6% fibre) displays both an antidiabetic and hypocholesterolaemic activity, sub fraction 'b' (52.8% protein and 7.2% saponins) has a hypolipidaemic effect since it reduces elevated cholesterol and triglyceride levels in diabetic dogs. This latter sub fraction was further fractionated into two fractions, 's' which contain the saponins (22.2%) and sub fraction 'p' containing the proteins (70.5%). The 'p' sub fraction had no effect on the cholesterol and triglyceride levels in the diabetic dogs.

Stark and Madar (1993) found the hypocholesterolaemic effect of saponin-rich isolate of an ethanol extract of fenugreek seeds. They observed that the saponin-rich fraction lowered plasma cholesterol levels in hypocholesterolaemic rats by 18-26%. The ethanol extract inhibited bile acid absorption in inverted segments of rat intestine in a dose dependent manner. This suggests that an interaction may be occurring in the digestive tract between bile acids and saponins. It has been proposed that the micelles are formed from bile acids and saponins, which are not available for absorption due to their large molecular size. This mechanism has been proposed for other saponin containing foods, such as soya, which also have hypocholesterolaemic properties (Sidhu and Oakenful, 1986).

Sauvaire et al. (1991) examined the transformation of steroid saponin-rich sub fractions of fenugreek seeds during their passage through the digestive tract, to determine the relative contribution of saponins and/or diosgenin and their steroid sapogenins to the hypocholesterolaemic effect of fenugreek seeds. In this study, faecal samples from alloxan diabetic dogs fed the fenugreek sub fractions were analyzed by capillary gas chromatography/mass spectrometry for the presence of sapogenins. Their result suggests that saponins, are in part (about 57%), hydrolyzed into sapogenins (diosgenin, smilagenin, gitogenin) in the digestive tract. The authors concluded that saponin hydrolysis does occur, presumably in the stomach and/or in the proximal small intestine. Since, hydrolysis was incomplete; saponins may be implicated, alone or together with sapogenin, in the observed hypocholesterolaemic effect on the fenugreek seeds.

Human and animal studies point to saponins having beneficial effects in lowering lipid status including cholesterol. The hypocholesterolaemic effects appear to be associated primarily with reduced intestinal reabsorption of cholesterol and bile acids. This activity has been linked to the saponin and sapogenins present in the fenugreek seeds. Fenugreek as a dietary supplement may thus hold promise in controlling serum cholesterol and hence the potential to be an effective functional food. It is however, noteworthy that all studies concerning fenugreek involved the legume of South Asian origin. It is only recently, fenugreek has been a specialty crop in Western Canada. The predicted increase in cultivation of this legume crop in Canada comes from Dr Acharya's work at the Lethbridge Research Center (Agriculture Agri-Food Canada) with an objective to develop new forage cultivation for use in the Western Canada cattle industry. While the benefiting properties of the fenugreek grown in South Asia are well known (Madar and Stark, 2002), the properties of the legume of Canadian origin have remained entirely unexplained. The present study was undertaken to examine the cholesterol lowering potential of saponin, extracted from the seeds of Canadian grown fenugreek.

1.2. Hypothesis and Objectives

It is hypothesized that fenugreek of Canadian origin is a rich source of saponin and that saponin binds cholesterol and bile acid, the property that is attributable to lower plasma cholesterol level.

Using a fenugreek variety (Amber), grown in Lethbridge Research Centre, Agriculture Agri-Food Canada, Alberta this hypothesis was tested with the following objectives:

1. To extract, purify, identify and characterize saponin present in the fenugreek seed.
2. To examine cholesterol and bile acid binding capability of the saponin extracted from fenugreek seed.

1.3. References

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CHAPTER 2

Extraction and Purification of Saponin from Canadian Fenugreek Seeds

2.1. Introduction

Saponins in seeds of fenugreek have been of interest for many years, primarily because diosgenin has been found to be a precursor for the synthesis of pharmaceutical steroids. There have been numerous studies attempting to analyze the diosgenin content of fenugreek seeds (Marker et al., 1947; Fazli and Hardman, 1971; Hardman and Jefferies, 1972; Mahato et al., 1982; Gupta et al., 1984, 1985; Petit et al., 1995). Since saponins have relatively large molecular weights and are highly polar, their isolation poses a challenge. The presence of complex mixtures of compounds, differing either in the nature of aglycone or the sugar part involving nature, number and position of attachment of the monosaccharides poses further challenges in isolating purified saponins (Hostettmann and Marston, 1995). Different methods and techniques have been employed for the purpose of isolation and purification of fenugreek saponin. In general, the extraction has been done in two stages: defatting of seeds followed by solvent extraction. Commercial fenugreek seed powder of Moroccan origin was defatted with petrol and then extracted with methanol until the extract became colorless (Hardman et al., 1980). Dried seed sample was extracted with hexane for 10 hr. The hexane extracted seeds were then extracted for saponin with methanol for 24 hr (Sharma, 1986). Sauvaire et al. (1991) defatted the fenugreek seeds with hexane and then fractionated the defatted seeds. The fraction

containing cotyledons and axis were extracted with isopropanol and water (70:30, v/v). The extract was then lyophilized; lyophilized extract contained 22.2% saponin. In another attempt, the ground fenugreek seeds were defatted in light petroleum ether, and dried at room temperature. The defatted seeds were then extracted for saponin with absolute ethanol (Stark and Madar, 1993). Petit et al. (1995) used a different technique, in which at first the fenugreek seeds powders were extracted with ethanol and water (25:75, v/v) at room temperature. The filtrates were concentrated in rotary evaporator. This extract was then defatted with hexane. In another saponin extraction procedure the dried ground fenugreek seeds were extracted for 24 hr with petroleum sprit (40-60°C) at room temperature. The defatted seeds were further extracted for saponin by boiling methanol and the crude saponin was estimated to be approximately 5.5% of the seeds (Al-Habori et al., 1998). Powdered fenugreek seed samples were heated under reflux for 3 h with petroleum ether (60-90°C) and the defatted seed samples were then extracted for saponin under reflux for 4 h with anhydrous ethanol (Zhao et al., 2002). In a recent study, Saxena and Shalem (2004) have used petroleum ether for the defatting of the fenugreek seeds and have used methanol for the extraction of the saponin from the defatted seeds.

All of the above extraction techniques are very different from each other. The use of any one solvent was unable to extract maximum lipids and lipid-like compounds from the seed powder. However, hexane extraction is an easy and efficient technique for the elimination of lipids and volatile substances (Damjanovic et al., 2003), while the complete elimination of the solvent especially the hexane from the residue is not always possible (Fred et al., 2004). Hence, special care should be

taken for the maximum elimination of the hexane from the residue as ingestion of hexane may cause health hazards including abdominal pain and nausea.

Hardman et al. (1980) used column chromatography technique for the purification of the saponin. The dried methanolic extract was dissolved in water and washed with water saturated with n-butanol. The aqueous phase was purified by column chromatography first on silica gel eluted with $CHCl_3$-MeOH-H_2O (13:6:1) and then again on ion exchange resin and eluted with methanol. Final purification has been done on acidic alumina column eluting with n-BuOH-EtOH-H_2O (5:1:1). Petit et al. (1995) claimed 90% purity of their extract using their technique of purification. In this technique, the concentrated ethanolic extract of the fenugreek seeds were defatted with hexane and freeze-dried. After solubilization of the dry extract with a mixture of water and ethanol, removal of gum was carried out by precipitation with alcohol. The hydro-alcoholic extract was then passed onto an ion exchange resin, H^+ form, to trap the basic compounds. The eluate of the chromatography was dialyzed and purified by gel filtration with Sephadex LH20. Saxena and Shalem (2004) reported another method of purification of saponin. The concentrated methanolic extract of the defatted seeds was shaken with $CHCl_3$ and filtered. The residue was taken up in H_2O and extracted with n-BuOH. The n-BuOH extract was concentrated under vacuum and then chromatographed over alumina with benzene.

All of the above techniques are complex and used some chemicals which are not food-safe. There is an essential requirement of a state-of-the-art technique for the purification of fenugreek saponin, which should be economical and useful to the industry.

Different methods are applied for the detection of the steroidal sapogenin in fenugreek. The widely used methods are blood hemolysis, color reaction, infrared spectrophotometric analysis and thin layer chromatography (Fazli and Hardman, 1968; Hardman and Jefferies, 1972; Hardman and Fazli, 1972a; Dawidar and Fayez, 1972). The extract of fenugreek seeds has also been analyzed by UV spectrophotometry (Sanchez et al., 1972; Sharma and Kamal, 1982), HPLC techniques (Ortuno et al., 1998) and by GC techniques (Bohannon et al., 1974; Brenac and Sauvaire, 1996; Taylor et al., 1997). Prior to an *in situ* hydrolysis of the saponin by aqueous hydrochloric acid and $CHCl_3$ extraction, the measurement of the specific spirostan absorption and calculation of the absorbance of the bands at 915 cm^{-1} and 900 cm^{-1} enables the determination of the 25β- and 24α- forms separately, with a 3-10 % overall error for individual C_{25} epimers and 3-5 % for total sapogenin (Brain et al., 1968). The IR spectrophotometric analysis of the crude extracts was later shown by Hardman and Jefferies (1972) to give high values, and was replaced by column chromatography preceding IR analysis. This method removes sterols, steryl esters, spirostadienes and dihydroxysapogenins, such as gitogenin from the fraction containing diosgenin and yamogenin and it has been further improved (Jefferies and Hardman, 1976).

The present study was undertaken to develop a simple and convenient method for the extraction and purification of the saponin from the fenugreek seeds.

2.2. Materials and Methods

2.2.1. Plant Materials

The seeds of fenugreek (cultivar: Amber) were collected from Lethbridge Research Center, AAFC, Alberta, Canada. Before grinding, the seed materials were sorted by hand to remove vegetative materials and dead seeds. The seeds were then dried for 48 h at room temperature. The seeds were ground with a heavy-duty kitchen grinder and sieved (mesh size 200 μm). The seed materials remained after sieving were grounded and sieved again. This step was repeated several times until all the seed materials became powder. The ground seed materials were dried at room temperature for 72 h. After drying the seed powder was kept in polythene bags and stored in a freezer at -20° C.

2.2.2. Chemicals

Petroleum ether (BP 60° C), hexane, methanol and water used in different steps of the experiments were purchased from Fisher Scientific (Nepean, ON, Canada). All the chemicals used were HPLC grade unless otherwise mentioned. The ethanol used for the saponin extraction was purchased from Commercial Alcohols Inc. Winnipeg, Manitoba, Canada. Deionized water was obtained from a Milli-Q water purification system (Millipore Co., Bedford, MA).

2.2.3. Lipid Extraction

2.2.3.1. Defatting with petroleum ether and hexane

The ground seed (25 g) was taken in double thickness cellulose extraction thimbles (Whatman, Florham Park, New Jersey, USA) and defatted in Soxhlet apparatus in two stages.

The seed sample was extracted with hexane for 16 h. The meal to solvent ratio was 1:5. After the extraction, the defatted seeds were air dried at room temperature for 3 days to evaporate the hexane from the residue.

The hexane extracted seed powder was then extracted for 8 h with petroleum ether (BP 80° C) and the meal to solvent ratio was 1:3. The extracted seed sample was then dried at room temperature for 3 days. After three days of drying the defatted seeds were weighted and stored in glass bottle at room temperature for further studies. All the steps of the defatting had been done under a fume hood.

2.2.3.2. Supercritical fluid extraction

Supercritical fluid extraction method has also been performed for the defatting of the fenugreek seed powder. This supercritical fluid extraction was carried out at the facility of Newlyweds Foods (Norac Division), Edmonton, AB, Canada. The extraction conditions were 500 bar/60°C with two-step fractionation of 120 bar/60°C

and 40 bar/60°C, respectively. The CO_2 mass flow rate was 31 kg/hr and CO_2 usage was 40:1 with respect to raw material.

2.2.4. Extraction of Saponins

Different extraction methods have been tried to find the best method for the saponin extraction from defatted fenugreek seed powder. The seed powder defatted with hexane and petroleum ether has been used for saponin extraction.

2.2.4.1. Procedure – 1

The dried defatted seed powder (50 g) has been extracted at room temperature with 150 ml of 80% ethanol. The seed powder was taken in the thimble and kept straight inside the beaker containing the solvent for extraction. The beaker was then tightly covered with a piece of aluminum foil and placed on a horizontal shaker. The seed powders were extracted for 24 h. At the end of the extraction, the solvent has been collected and evaporated in rotary evaporator.

2.2.4.2. Procedure – 2

This method was similar to procedure – 1. The only difference was the solvent quality. The solvent used was 100% ethanol instead of 80% ethanol to test the efficiency of the two different solvent in saponin extraction from defatted fenugreek seeds.

2.2.4.3. Procedure – 3

In the third method of extraction, the defatted seed powder was extracted using a Soxhlet apparatus. The seed powder was extracted with 100% ethanol for six hours and the solvent to meal ratio was 1:5. After the extraction the solvent has been collected and evaporated using a rotary evaporator.

2.2.4.4. Procedure – 4

Weighed quantity (50 g) of defatted seed sample was transferred into a conical flask fitted with west condenser and extracted with 80% ethanol. The meal to solvent ratio was 1:5 and the extraction time was 4 hr. The mixture was heated at 60° C in a water bath with constant stirring using a magnetic stirrer. After 4 hr of extraction the mixture was kept at room temperature overnight and filtered through filter paper. The

residue was again extracted for a second time with 80% ethanol. The meal-to-solvent ratio was 1:4. The other physical condition was similar to those in the first extraction. After the completion of the extraction, the mixture was kept overnight at room temperature and filtered through filter paper. The extracts were deep brown in color. Both extracts were combined and concentrated in a rotary evaporator at a maintained temperature of 50° C. The concentrated extract was then freeze dried and stored in a glass bottle at room temperature.

2.2.5. Purification

Different purification methods had been employed to find an efficient and easy to use technique for saponin purification. The extracts collected employing the Procedure – 4 has been used for testing different purification methods for fenugreek saponins.

2.2.5.1. Purification procedure– 1

This method is based on Hardman et al. (1980). The dried extract was dissolved in water and washed with n-BuOH saturated with water. The solution of the extract was taken in a separatory funnel and mixed with equal volume of n-BuOH saturated with water. Solutions were mixed properly by hand shaking for about 10

min. The separatory funnel was then kept on the funnel holder and allowed to stand overnight. This step was repeated for two more times. The aqueous phases from three separations were combined and evaporated to get a semi-purified extract. The substances dissolved in butanol were collected as butanolic extract, evaporated to dryness and tested by TLC for the presence of saponin. This dried aqueous extract was then partially purified on silica gel column with an eluting solvent ($CHCl_3$-MeOH-H_2O, 13:6:1).

2.2.5.2. Purification procedure – 2

The principle of this method is the elimination of the non-saponin constituents of the extract. This method has been developed by Gurfinkel and Rao (2002) for the purification of the legume saponins. The dried extract (2.5 g) of the fenugreek seed was extracted with acetone (25 ml) at room temperature. The mixture was then centrifuged at 2000 g for 30 min. The acetone extraction was repeated three times. The residue after the centrifugation has been collected as a partially purified extract. The acetone parts from the three extractions were combined together and evaporated which yielded a sticky brown substance. Residue as well as the brown substances was both tested for the presence of saponin by TLC.

2.2.5.3. Purification procedure – 3

An aliquot (1 g) of the freeze-dried crude extract was dissolved in HPLC grade water and loaded on to a C18 Sep-Pak cartridge (Waters Corporation, Milford, MA, USA). Before the loading of the samples, the Sep-Pak cartridges were preconditioned with 5 ml of methanol and 10 ml of HPLC grade water. Special care had been taken to avoid drying of the Sep-Pak. After the preconditioning the extract solution was slowly (1 ml/min) loaded in the Sep-Pak cartridge. To maintain constant flow rate (2 ml/min), the extraction manifold (Supelco, Bellefonte, PA, USA) has been used. After the sample loading, the cartridge was washed with HPLC grade water and checked for uniform loading. The cartridge was then eluted with 30% methanol. The elution with 30% methanol was continued until no color in the elution solvent has been observed. Finally the saponins were eluted from the Sep-Pack with 70% methanol. The 70% methanol eluate was concentrated in a rotary evaporator maintaining the temperature at 50° C and the concentrated sample was finally freeze dried and stored at room temperature in a desiccator until further used.

2.2.6. Identification of fenugreek saponin

Thin layer chromatography (TLC) The TLC was performed on Silica gel G plate (Fisher Scientific, Nepean, ON, Canada). TLC method used was similar as developed by Konishi et al. (1984) and Petit et al. (1995) for the identification of

furostanol and spirostanol saponins. The purified saponin (10 mg/ml) was dissolved in 70% methanol and 5 µl samples were spotted on 20 cm X 20 cm TLC plate. The plate was developed with $CHCl_3$ – CH_3OH – H_2O (65 : 42 : 10, v:v:v). For the detection of different forms of steroidal saponins two kinds of visualizing agents has been used separately. The detection of furostanol saponins was done by spraying Ehrlich's reagent (3.2 g of p-dimethylamino benzyldehyde in 60 ml of 95% ethanol and 60 ml of 12 N HCl) and heated at 110°C for 5 min to develop bright red spots. All the organic compounds became dark brown when sprayed with 10% sulfuric acid in MeOH, followed by heating at 110°C for 5 min.

2.2.7. Statistical analysis

Data obtained were subjected to analysis of variance using general linear model procedure in SAS (Statistical Analysis System, Version 7.0, SAS Institute Inc., Cary, NC, USA). Means of different procedures were compared Tukey's studentized range test using SAS.

2.3. Results and Discussion

2.3.1. Lipid Extraction

Different extraction processes were helpful to eliminate possible interfering compounds with saponin of fenugreek seeds. Most of the lipids and lip soluble compounds have been extracted and eliminated by two successive extractions with hexane and petroleum ether. The total lipid extracted from fenugreek seeds using this method was calculated to be 7.17% of the whole seeds.

For the purpose of lipid extraction the super critical fluid extraction method was also tested. The supercritical solvent was able to extract 5.31% of lipid and lipid related compounds from the fenugreek seeds (Table 2.1). Further extraction of these supercritical fluid extracted seeds with hexane eliminated 0.92% of lipids and lipid-like compounds. The supercritical fluid extraction may not be suitable method for the extraction of lipid and lipid-like compounds from the fenugreek seeds. However, before drawing any final conclusion a detailed study with fenugreek seeds using the supercritical fluid extraction is required. Different parameters need to be tested elaborately.

Table 2.1. Effect of defatting of lipid and lipid like compounds

Procedure	Extract (%)
Hexane and Petroleum Ether (Lab scale extraction)	7.17 ± 0.39
Supercritical Fluid Extraction (Pilot scale extraction)	5.31 ± 0.26

$p<0.05$

Till a suitable technique found for the defatting of the fenugreek seeds; the hexane and petroleum ether are considered as a most reliable solvent for lipid extraction. Hexane is not considered as health friendly and a complete elimination of the hexane from the seeds is impossible. Understanding this problem, petroleum ether has been used as a second solvent on hexane extracted seeds for the extraction of more lipids and also the residual hexane from the fenugreek seeds.

2.3.2. Extraction of Saponins

Saponins are one of the polar compounds present in the fenugreek seeds. Different solvents as well as different physical conditions have been tried to find a suitable method for the saponin extraction from the defatted fenugreek seeds.

In procedure – 1, ethanol (80%) was used as a solvent of extraction. The physical conditions were room temperature and agitation of the solvent. The extraction yield was 6.42% (Table 2.2).

In procedure – 2, the solvent was replaced by 100% ethanol. The 80% ethanol has extracted a significant higher amount of extract than 100% ethanol (0.31%) from the fenugreek seeds.

In procedure – 3, the seeds were extracted with 100% ethanol using Soxhlet apparatus for 6 hrs. This extraction yield was 2.66% from the defatted fenugreek seeds (Table 2.2).

In comparison with other methods tested, highest amount of extract was extracted from defatted fenugreek seeds using the procedure – 4. In this method 80% ethanol was used with agitation maintaining a temperature of 60° C. The total extract estimated was 20.56% (Table 2.2).

Table 2.2. Effect of various extraction procedures on extraction of saponin

Procedures	Extract (%)*
Procedure 1 (80% EtOH, 24 hr)	$6.42^{a} \pm 0.20$
Procedure 2 (100% EtOH, 24 hr)	$0.31^{b} \pm 0.01$
Procedure 3 (100% EtOH, 6 hr, Soxhlet apparatus)	$2.66^{c} \pm 0.18$
Procedure 4 (80% EtOH, 60° C, 4 hr)	$20.56^{d} \pm 0.59$

* dry weight basis; Values are mean of five observations; Mean values superscripted with different letters are significantly different at $p<0.05$

Ethanol is a better solvent over methanol for the extraction of the saponins from the defatted fenugreek seeds as during the extraction with methanol, the furostanol saponins form 22-OCH_3 derivatives. The 22-hydroxyfurostanols can also be obtained by the extraction with solvents like pyridine or by treating the methoxylated artifacts with boiling aqueous acetone (Konishi and Shoji, 1997). Fenugreek saponins are of furostanol type and therefore, ethanol has been used as an extraction solvent for saponins from defatted fenugreek seeds.

At the time of saponin extraction, 80% ethanol was found to be most suitable over different concentrations of ethanol (60%, 70%, 90%) tried. To evaluate the solvent extraction capability different concentration of ethanol (60%, 70%, 90%)

were used for extraction using extraction procedure – 4. The extracts were dried and weighted to evaluate the suitability of the extraction solvent and the maximum quantities of extracts have been recovered by using 80% ethanol.

2.3.3. Purification of saponins

The purification procedure -1, which was based on Hardman et al (1980) and using that method the separation of saponins was not possible. Both butanol and water phase showed the presence of saponins in TLC. This method was not found to be suitable for the purification of fenugreek saponin.

The 2nd purification procedure tried (as developed by Gurfinkel and Rao, 2002), showed the presence of other substances when tested using TLC. Furthermore, acetone used for this technique is considered hazardous chemicals for human and animal health.

In the procedure – 3, the purification was carried out by employing C18 sep-pak with different concentrations of methanol as an eluting solvent. The yield of the purified extract was calculated as 22.42% of the extract.

Employing this technique an extract was obtained containing concentrated forms of saponins as demonstrated in TLC.

The purification was carried out by using C18 sep-pak as this is a suitable solid support for the elimination of most of the proteins and carbohydrates and pigments (Miniati and Montanari, 1998; Collu et al., 1999; Megias et al., 2004). This

one step procedure virtually makes the extract more purified and concentrated in saponins.

The purification is a one step method in which the extract has been purified with the elution of water and 30% methanol. After loading of the extract the water has been used to wash all the hydrophilic compounds and then the washing with 30% ethanol slowly wash off other more polar compounds. The 70% methanol elutes the saponins adsorbed with the C18 adsorbent. Each fraction was monitored by TLC to determine any possible loss of saponin during purification. The evaporation of the methanol at low temperature (35°C) and ultimate drying in freeze dryer eliminates the chance for the formation of the other unwanted artifacts.

The visualization with the spraying of Erlich's reagent (especially for furostanol saponin) and with sulfuric acid and methanol the TLC of extract and purified extract clearly demonstrated that the purified extract is devoid of interfering substances. With the same concentration of both the extract and purified extract the bands of saponins in purified extract were more prominent. It also demonstrated that the saponin present in the Canadian variety (Amber) of fenugreek is of furostatic form.

It appears that the fenugreek may best be defatted with hexane and petroleum ether. For the extraction for saponins from the defatted seeds, the 80% ethanol is the most suitable solvent when used with agitation and a temperature at 60°C. The C18 is the most economical and easy to use adsorbent for the purification of the extract to get concentrated saponins (Figure 2.1). In the following chapters, filtered and dried ethanolic extract of fenugreek seeds containing saponin, soluble fibers, simple sugars,

and other polar compound like proteins, will be considered as "CRUDE EXTRACT". The latter which has been further purified through C18 column and rich in saponin and devoid of other compounds like soluble fibers, simple sugars and proteins will be considered as "PURIFIED EXTRACT".

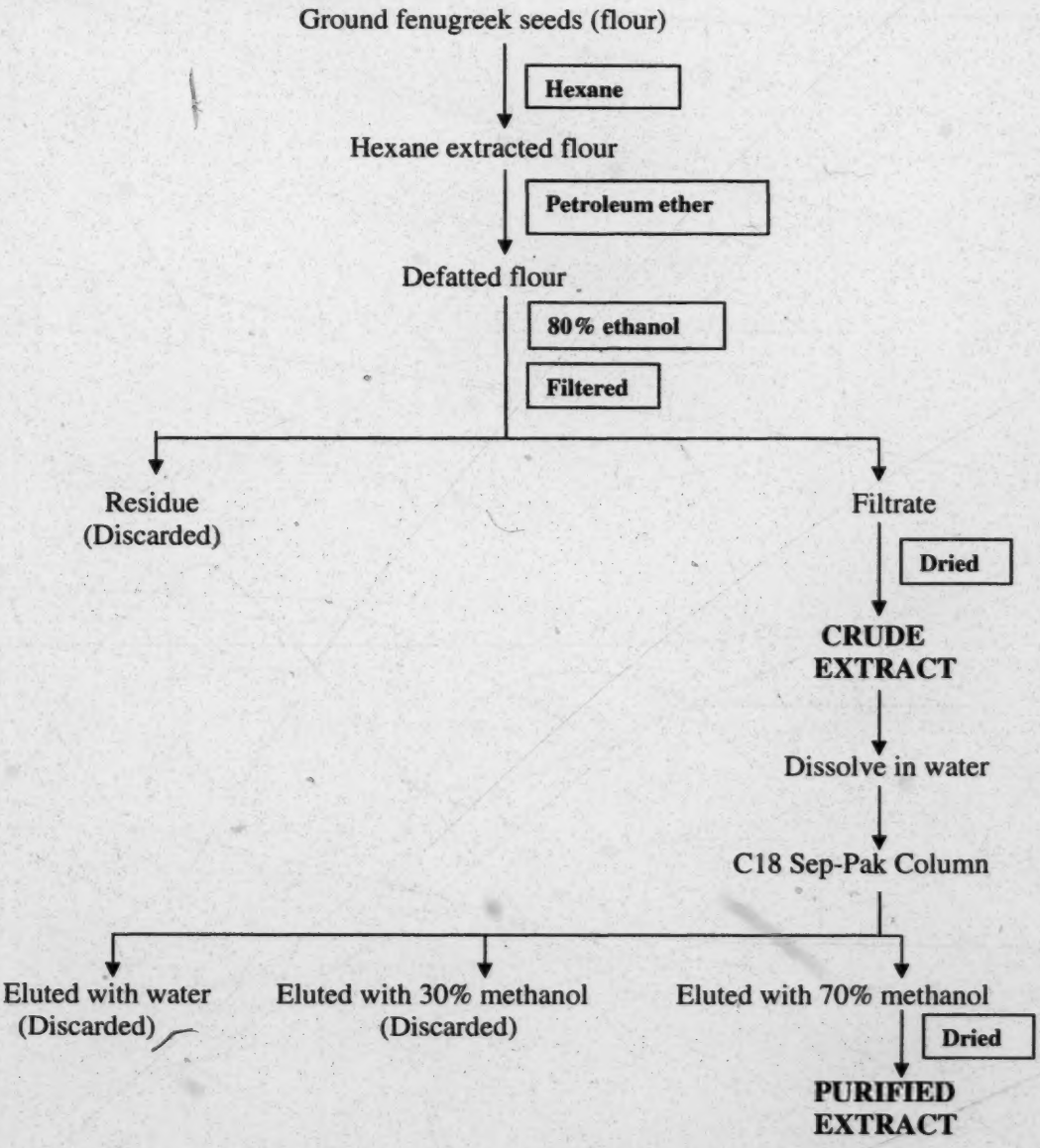

Figure 2.1. Schematic representation of the extraction and purification process

2.4. References

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CHAPTER 3

Analysis of Crude Extract and Purified Extract

3.1. Introduction

Saponin consists of sapogenin (or aglycone) and carbohydrate (or glycone). Depending on the type of sapogenin present the saponins are classified into three major classes: 1) triterpene 2) steroid and 3) steroid alkaloids (Figure 3.1).

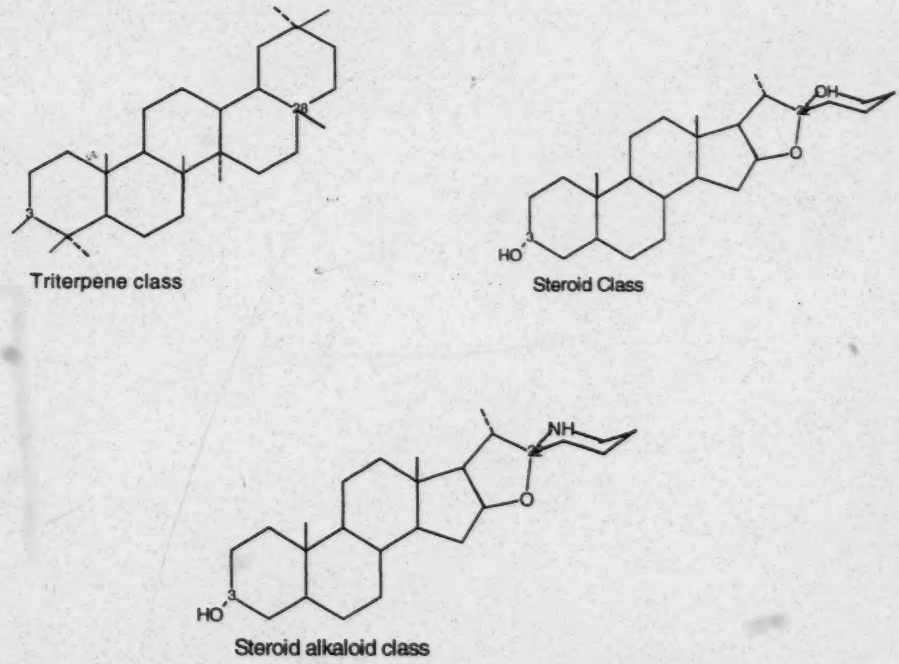

Figure 3.1 Chemical structures of different sapogenins

Saponins contain one or two carbohydrate chains attached with the sapogenins. These are grouped (Figure 3.2) into monodesmosidic (one carbohydrate chain attached) or bidesmosidic (two carbohydrate chains attached).

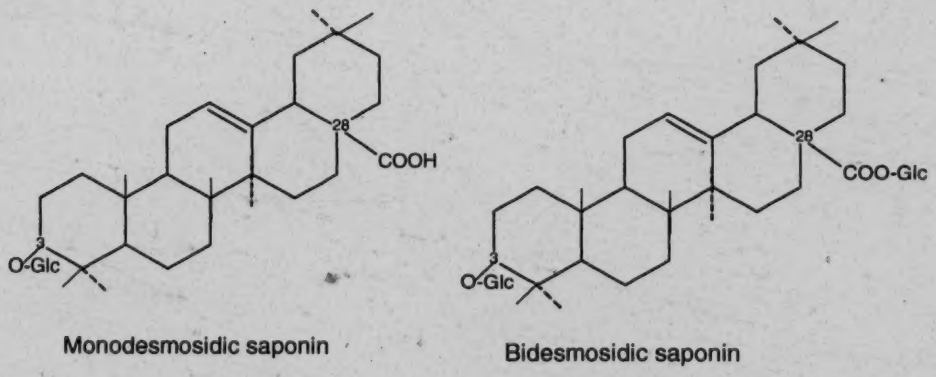

Figure 3.2. Chemical structures of saponins

The major sapogenin in fenugreek seeds is diosgenin. In the seed, diosgenin is only present in embryo, but it is absent from the testa and endosperm (Fazli and Hardman, 1968); it is also present in other parts of the plant (stems, leaves etc.) but in very minor quantity (Hardman and Fazli, 1969; Varshney et al., 1980). The diosgenin has considerable economic importance in the pharmaceutical industry as a starting material for the partial synthesis of oral contraceptives and other medicinally useful steroids. The tubers of the *Dioscorea* are the traditional source of diosgenin. However, an increased demand for raw steroids has led the industries to look for an alternative source of diosgenin and other precursors and fenugreek has thus been considered as an alternative source of diosgenin (Marker et al., 1947; Fazli and

Hardman, 1968; Bhatnagar et al., 1975) It has also been proposed by Hardman (1980) that fenugreek could be grown widely as a source of diosgenin with its equally important epimer, yamogenin.

However, there are different contradictory reports regarding the sapogenin quality and quantity of fenugreek. The presence of steroidal substances in fenugreek seeds was first reported in 1919 by Wunschendroff. The steroidal sapogenin in the alcoholic extract of fenugreek seeds was subsequently reported by Soliman and Mustafa (1943). On the same year, Marker et al. (1943) extracted the same sapogenin from the seeds of the fenugreek and identified it as diosgenin. Later, Marker et al. (1947) described the alcoholic extract of fenugreek seeds as a mixture of sapogenins and they identified the sapogenins as diosgenin, gitogenin and tigogenin. These findings were confirmed by Soliman and Mustafa (1949), but they have not reported the presence of tigogenin in the extract. In addition, Soliman and Mustafa (1949) isolated another sapogenin from the extract, which they named as trigonellagenin. Bedour et al. (1964) isolated 25α-spirosta-3, 5-diene besides the three sapogenins reported by Marker et al. (1943); however, failed to find trigonellagenin.

In 1971, Fazli and Hardman isolated one more sapogenin, the epimer of diosgenin, neotigogenin from the fenugreek seeds of West Pakistan and Moroccan origin. Trigonellagenin, found by other workers is considered as the mixture of diosgenin and yamogenin. They also detected trace a amount of tigogenin in Moroccan seeds only. The total sapogenin content of the whole seed of fenugreek was 1.27% for the West Pakistani seeds and 1.50% for the Moroccan seeds.

Dawidar et al. (1973) examined the fenugreek seeds of Egypt origin and isolated and reported for the first time the presence of the neotigogenin. More sapogenins were reported in subsequent years (Knight, 1977) and these include yuccagenin, liagenin, and neogitogenin along with diosgenin, yamogenin, tigogenin, neotigogenin, and gitogenin.

Depending on the geographical origin of the fenugreek seeds, the sapogenin content varied from 0.8 to 2.2% expressed on a moisture-free basis. The sapogenin present in the Moroccan variety has been quantified and found to contain 2.12% of diosgenin (Fazli and Hardman, 1968).

Sapogenins (diosgenin and yamogenin) have been isolated and quantified from 12 commercial fenugreek seed samples collected in different locations of India (Puri et al., 1976). The diosgenin plus yamogenin contents of these samples ranged from 0.6 to 1%, while the diosgenin to yamogenin concentration ratio remained the same (3:2) in all the cases. Similar variability in diosgenin levels in seeds of fenugreek collected from various geographical regions of India was also reported by others (Sharma and Kamal, 1982); the diosgenin content varied from 0.33 to 1.9%. Taylor et al. (2002) observed variation in diosgenin contents among 10 accessions of fenugreek seeds produced in different locations in western Canada. The diosgenin levels from mature seeds ranged from 0.28 to 0.92%.

There is no free sapogenins present in fenugreek seeds. Generally, sapogenins occur in the fenugreek plant as furostanol glycosides from which spirostanol glycosides are secondarily formed (Sauvaire and Baccou, 1978). These glycosides

occur in the embryo, but absent from other parts of the seed like seed coat, testa and endosperm.

Sapogenins are released only after enzymatic or acid hydrolysis (Blunden and Hardman, 1963). The sapogenins available following the process of acid hydrolysis of fenugreek seeds are mainly the monohydroxysapogenins (diosgenin, [25R-spirost-5-en-3β-ol] and its 25-S- epimer, yamogenin). About 10 % of their weight is a mixture of the two corresponding 5-α-saturated monohydroxysapogenins, tigogenin and neotigogenin. In addition to these four sapogenins, there are very small percentages of each of their corresponding 2-hydroxy derivatives, namely yuccagenin, lilagenin, gitogenin and neogitogenin, respectively (Cornish et al., 1983). Sarsapogenin and smilagenin have also been isolated from the hydrolyzed seeds (Gupta et al., 1986b).

Fenugreek seeds mainly contain steroids of the 25-S series, but during acid hydrolysis some of these are converted into the 25-R-spirostanes (Bogacheva et al., 1976b).

Yoshikawa et al. (1997) isolated six furostanol saponins called trigoneosides Ia, Ib, IIa, IIb, IIIa and IIIb from fenugreek seeds of Indian origin, together with two known saponins, glycoside D and trigofoenoside A. In the following year, the same group of researchers (Yoshikawa et al., 1998) in isolated seven new furostanol saponins from Indian fenugreek seeds, called trigoneosides IVa, Va, Vb, VI, VIIb, VIIIb, IX along with known furostanol saponins, compound C, glycoside F and trigonelloside C. There were six furostanol types of steroidal saponins called trigoneosides Xa, Xb. XIb, XIIa, XIIb and XIIIa isolated from the seeds of Egyptian fenugreek together with six known furostanol-type steroid saponins: trigoneosides Ia,

Ib, Va, glycoside D, trigonelloside C, and compound C (Murakami et al., 2000). The structures of the trigoneosides (e.g. Xa, Xb. XIb, XIIa, XIIb and XIIIa) were determined on the basis of their chemical and physiochemical properties.

There are seven spirostanol saponins called graecunins H-N, which are glycosides of diosgenin with different sugar moieties; these glycosides have also been isolated from fenugreek seeds. Graecunins H, I, J and K contain varying amounts of glucose and rhamnose, whereas graecunins N contains glucose, arabinose, xylose and rhamnose (Varshney and Begs, 1978). Another saponin, named fenugrin B, has also been obtained from the fenugreek seed. This compound on acid hydrolysis, gave diosgenin and sugars: glucose, arabinose and rhamnose (Gangrade and Kaushal, 1979).

There are several reports on the fenugreek saponin but reports are scanty on fenugreek extracts. There has not been any report on fenugreek extract of Canadian origin. The characterization of the extract and purified extract is important before further studies can be done on physiological properties of the crude and purified extracts. In this chapter, crude extract and purified extract were analyzed for the characterization for their chemical constituents. Efforts were made to test and quantify the presence of soluble and insoluble fiber, sapogenins and the probable carbohydrates present attached to sapogenins.

3.2. Materials and Methods

3.2.1. Determination of soluble and insoluble dietary fiber in crude extract and purified extract

This method AACC method 32-07 is the simplified modification of the AACC soluble/insoluble dietary fiber method (for oat products), 31-21. Duplicate samples of crude extract as well as purified extract of fenugreek seeds were used for the determination of the soluble and insoluble fiber. Samples were weighed (1.000 ± 0.005 g) accurately and placed into the tall-form beaker.

3.2.1.1. Chemicals

All the reagents were analytical grade and obtained from Fisher Scientific (Nepean, ON, Canada) unless otherwise mentioned. The ethanol used for the analysis was purchased from Commercial Alcohol Inc, Winnipeg, Manitoba, Canada. Deionized water was procured from Milli-Q water purification system (Millipore Co., Bedford, MA). α-Amylase, protease and amyloglucosidase (all enzymes were stored at 0 – 5 °C) and acid washed/pre-ashed celite were supplied by Megazyme International Limited. Tris (hydroxymethyl) aminomethane (TRIS) was obtained from Sigma Chemical Co, St Louis, MO, USA.

3.2.1.2. Procedure

TRIS/HCl solutions (40 ml) were added in each beaker and stirred on a magnetic stirrer until the extract is completely dispersed in the solution. 50 µl of heat-stable α-amylase solution was added in each beaker while stirring at low speed and then the beakers were covered with aluminum foil squares. They were then placed in a shaking water bath at 95 – 100 °C and incubated for 35 min with continuous agitation. After the incubation all the beakers were removed from the water bath and cooled to 60 °C.

Protease solution (100 µl) was added in each beaker, recovered with aluminum foil and incubated in shaking water bath at 60 ± 1°C, with continuous agitation for 30 min. While stirring the solutions in the beaker 5ml of the 0.561 N HCl solution was added. pH was adjusted between 4.1- 4.8 with the help of 5% NaOH and 5% HCl solutions.

Amyloglucosidase solution (200 µl) was added while stirring on magnetic stirrer and again covered with aluminum foil. The beakers were then incubated in shaking water bath at 60°C for 30 min, with constant agitation.

The crucibles containing celite were weighed accurately nearest to 0.1 mg. The celite of each crucible was wetted and the bed of celite in the crucible was redistributed using approximately 3 ml of deionized water. Suction was applied to crucible to draw celite onto fritted glass as even mat. For the determination of the insoluble fiber the enzyme treated mixtures were filtered through the crucible into a filtration flask. The residues were washed twice with 10 ml of preheated (70°C)

deionized water in each step. The filtrates were saved for the determination of the soluble dietary fiber.

The determination of the soluble fiber was done by a precipitation method. Ethanol (95% at 60°C) was added (4:1) to the filtrate collected from the previous step and the mixture was allowed to precipitate at room temperature for 60 min. The crucibles containing celite were measured accurately nearest to 0.1 mg. The celite of each crucible was wetted and the bed of celite in the crucible was redistributed using approximately 3 ml of 78% ethanol. Suction was applied to crucible to draw celite onto fritted glass as even mat. The filtrate-ethanol mixture was filtered through the crucible. The residues were washed with two 15 ml portions of 78% ethanol, 95% ethanol and acetone. Crucibles containing residues were dried in 103°C oven.

3.2.2. Determination of the sapogenins in the crude extract and purified extract

Gas Chromatography GC analysis was carried out with a Varian 3400 Gas Chromatography (Varian Inc., Walnut Creek, CA, USA) equipped with a Varian 8100 auto sampler and a flame ionization detector. Separations were performed on a RH-1 capillary column (30 m × 0.32 mm i.d., Rose Scientific Ltd., Edmonton, AB, Canada). The initial column temperature was set at 90 ° C and the column temperature was raised at 25 ° C/min to 270 ° C and then at 1 ° C/min to 290 ° C and held for 12.8 min. The carrier gas was helium, maintained at column head pressure of 22 psi. Initial injector temperature was 100 ° C and the increase at a rate of 150 ° C/min to 250 ° C

and held until the end of the run. The samples (1 µl) were injected. Peak integration was performed using Shimadzu Class-VP (version 4.2) Chromatography Laboratory Automated Software System (Scientific Software Inc., San Ramon, CA, USA).

The amount of diosgenin found in the crude extract and purified extract was obtained from the integrated peak area ratio of diosgenin to internal standard (6-methyl diosgenin). Relative response factor was determined using standard diosgenin and methyl diosgenin.

Acid Hydrolysis This method was employed following the procedure described by Taylor et al (2000) and Yang et al (2003). Sub samples of crude extract (10 mg) and purified extract (5 mg) were transferred to a test tube (100 X 16 mm) with Teflon-lined screw cap containing 3 ml of 70% 2-propanol containing 1M sulfuric acid. Each mixture was hydrolyzed by heating at 100°C for 2 hr on a heating block in dry bath. After completion of hydrolysis, the mixture was cooled down to room temperature and neutralized with 1M NaOH. The internal standard of 6-methyl diosgenin (200 µg) was added and mixed thoroughly by vortexing. The mixture was then extracted three times with HPLC grade chloroform (2 ml) for 10 min each time at room temperature and centrifuged at 500xg for 5 min. The combined chloroform extracts were dried under the flow of nitrogen. The residue was dissolved in toluene (1 ml) and analyzed in Gas Chromatography using the GC conditions as described before.

Quantitation Stock solutions were prepared by dissolving 50 mg of the diosgenin in 10 ml of ethanol and 20 mg of methyl diosgenin in 10 ml of the ethanol. The diosgenin solution (300 µl) was added in a screw caped test tube containing 2700 µl of the 70% 2-propanol containing 1M sulfuric acid and tightly capped with a Teflon lined cap. The mixtures were heated at 100 °C on a heating block and after 2 hr of heating they were cooled down to room temperature. The mixtures were then neutralized by adding 1M NaOH solution and 100 µl of the methyl diosgenin solutions were added in each tube. The mixture was then extracted three times with HPLC grade chloroform (2 ml) for 10 min each time at room temperature and centrifuged at 500xg for 5 min. The combined chloroform extracts were dried under the flow of nitrogen. The residue was dissolved in toluene (1 ml) and analyzed in Gas Chromatography. Relative response factors were calculated from the peak areas for diosgenin and methyl diosgenin. Relative response factors were used to calculate the amount of sapogenins present in crude extract and purified extract.

3.2.3. Determination of the sugars in crude extract and purified extract

For the determination of carbohydrates the samples were prepared by dissolving 65 mg each of crude extract or purified extract in 13 ml of 70% propanol with 1M H_2SO_4.

Carbohydrates were analyzed according to procedures described by Lien et al. (1997). The sample solutions (1 ml) were placed in (13 x 100 mm) test tubes with

Teflon lined caps and an additional 1 ml of the 70% propanol with 1M H_2SO_4 was added. The samples were then hydrolyzed at 110°C for 2 hr. After hydrolysis, 200 µl of internal standard (N-methylglucamine and myoinositol, at 10 mg/ml of distilled water for amino sugars and neutral sugars respectively) were added.

An aliquot of 1 ml of the hydrolysate was cooled in an ice-bath and made basic by adding 0.7 ml of concentrated (14.5 M) ammonium hydroxide. To 100 µl of this, 1 ml of freshly prepared sodium borohydride (30 mg/ml in anhydrous dimethylsulphoxide) was added and the reaction was allowed to continue for 90 min at 40°C. Excess sodium borohydride was decomposed with the addition of 200 µl of concentrated glacial acetic acid. 1-Methylimidazole (0.2 ml) followed by 2 ml acetic anhydride were added. The samples were vortexed and acetylation occurred at room temperature for 10 to 15 min. To this mixture was 5 ml of water added to decompose excess acetic anhydride and the mixture was cooled to room temperature. Alditol acetates were extracted with 4 ml of dichloromethane by vigorous shaking. The dichloromethane layer was washed twice with distilled water and evaporated to dryness under a flow of nitrogen.

Prior to analysis by GC, the samples were re-dissolved in 2 ml of dichloromethane and 1.0 µl of the sample was injected on to the column. Gas chromatographic analysis was carried out with a Varian 3400 Gas Chromatography (Varian Inc., Walnut Creek, CA, USA) equipped with a Varian 8100 auto sampler and a flame ionization detector. Separations were performed on a DB-17 capillary column (30 m × 0.25 mm i.d., J & W Scientific Inc., Folsom, CA). Initial injector temperature was 60° C, which was increased at a rate of 150° C/min to 270° C and

held for 20 min. The initial column temperature was raised at 30° C/min from 50° C to 190° C and maintained for 3 min, then increased at 5° C/min to 270° C and maintained for 5 min. The detector temperature was set at 270° C. Peak integration was performed using Shimadzu Class-VP (version 4.2) Chromatography Laboratory Automated Software System (Scientific software Inc., San Ramon, CA, USA).

3.2.4. Statistical analysis

Means and standard error of the means were determined using SAS (Statistical Analysis System, Version 7.0, SAS Institute Inc., Cary, NC, USA). Statistical comparisons of diosgenin, yamogenin and rest sapogenins levels were performed with general linear models procedures by one-way analysis of variance. A least significant difference test was used to evaluate differences among means.

3.3. Result and Discussion

3.3.1. Determination of the fiber in the crude extract

Fenugreek is a rich source of soluble dietary fiber (SDF). In this experiment, it has been determined whether the crude extract does contain any soluble or insoluble dietary fiber (IDF). It has been observed that extract contain some soluble fiber and a small amount of insoluble fiber. The soluble dietary fiber present in the crude extract

has been calculated to be 1.13%. The insoluble dietary fiber present in the crude extract is only 0.11% (Table 3.1).

Table 3.1. Estimation* of soluble and insoluble dietary fiber in crude extract

Type of fiber	Mean (g)	% of fiber (w/w)
SDF	0.0113 ± 0.004	1.13 %
IDF	0.0011 ± 0.0004	0.11 %

* Dry weight basis; Values are mean of four observations

3.3.2. Determination of the fiber in the purified extract

The experiment for the determination of the fibers in the purified extract showed no visible precipitation, meaning the absence of any soluble and insoluble fiber. The presence of fiber in the extract could enhance the binding of cholesterol and bile acid in the gut. Due to the presence of soluble dietary fiber, the crude extract could be more active than the purified extract in terms of cholesterol lowering potential.

3.3.3. Determination of sapogenins in crude extract and purified extract

The Thin Layer Chromatographic studies demonstrated the presence of saponins in crude as well as in purified extract (chapter 2). In this experiment the sapogenins present in saponins were extracted, identified and estimated. Using this

gas chromatographic method it was possible to make complete separations of the different sapogenins in crude extract and purified extract. Although 1M aqueous hydrochloric acid effectively hydrolyzed the steroidal saponin extracted with 80% aqueous ethanol from fenugreek seeds, the quantitative determination of diosgenin by capillary GC of hydrolyzed samples had certain limitations, especially in regard to control over artifact (diene) formation (Taylor et al., 1997). To control diene formation, 1M sulfuric acid in 70% 2-propanol was used for the hydrolysis of the extract and purified extract. Numbers of peaks representing dienes were relatively less in sulfuric acid treatment than the hydrochloric acid (Taylor et al., 2000).

A time study was done using 1M sulfuric acid in 70% 2-propanol. The diosgenin recovery was measured in three replicates and it was found that 2 hr of heating using a heating block was optimal. A similar result was also observed by Taylor et al., (2000).

Propanol was used as a solvent for the sapogenin extraction. Generally short chain alcohols and aqueous mixtures are commonly used for the extraction of sapogenin from fenugreek (Sauvaire and Baccou, 1978b; Sauvaire et al., 1996; Benichou et al., 1999). Propanol has been found to be a superior solvent for sapogenin extraction among various other solvents (ethanol, methanol, water etc) used (Taylor et al., 2000).

The analysis of the crude extract and purified extract (Figs 3.3 & 3.4 respectively) showed the presence of diosgenin in addition to other sapogenins. Identification of diosgenin and 6-methyl diosgenin was performed using pure diosgenin and 6-methyl diosgenin standard.

These chromatograms are similar to those presented by Taylor et al. (2000) and Puri et al. (1976) and their peak identifications for smilagenin, yamogenin neotigogenin, yuccagenin, gitogenin and neogitogenin have been followed. The diosgenin content is the highest among the different sapogenins present in crude extract as well as in purified extract (Table 3.2).

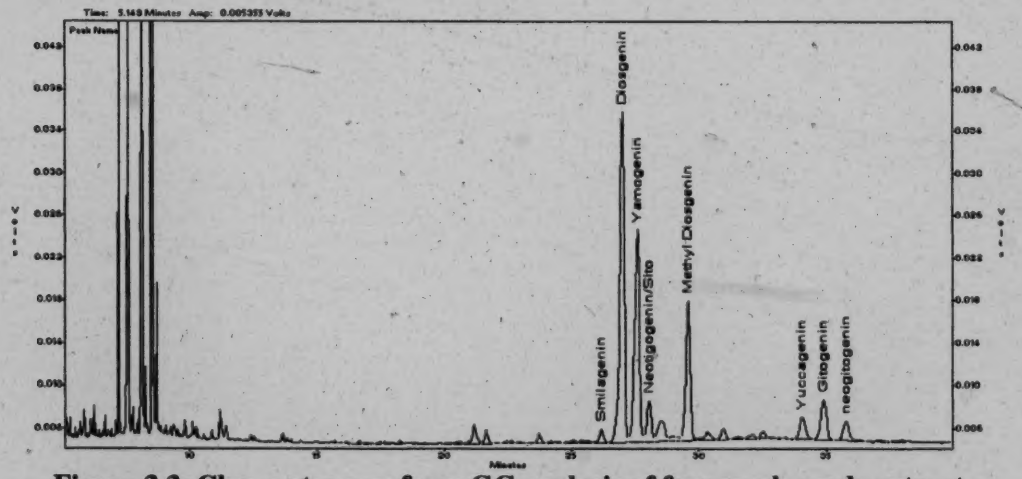

Figure 3.3. Chromatogram from GC analysis of fenugreek crude extract

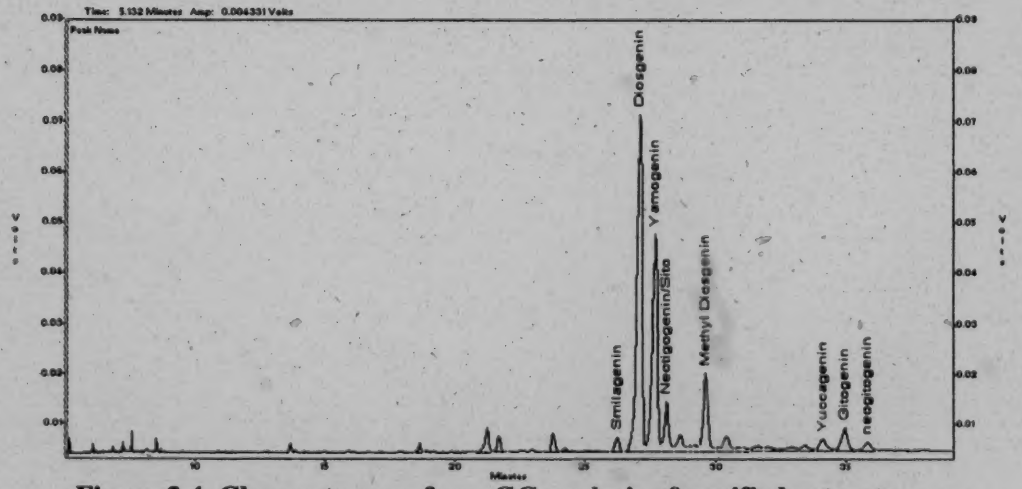

Figure 3.4. Chromatogram from GC analysis of purified extracts

Yamogenin, an epimer of diosgenin, is of equal utility to diosgenin as a starting material for steroid drug synthesis (Fazli and Hardman, 1971). From the industry perspective it is significant to estimate the yamogenin content in the crude extract and purified extract of fenugreek.

Yamogenin is the second highest sapogenin present in fenugreek seed crude and purified extracts. The yamogenin content in the extract is 16.64 µg/mg and in the purified extract it is 71.87 µg/mg (Table 3.2).

Table 3.2. Estimation* of sapogenins in crude extract and purified extract

Sapogenins (µg/mg)	Crude Extract	Purified Extract
Diosgenin	25.42 ± 1.58	109.08 ± 2.57
Yamogenin	16.64 ± 1.10	71.87 ± 1.71
Other Sapogenins (Smilagenin, Neotigogenin, Yuccagenin, Getogenin, Neogitogenin)	12.68 ± 0.81	34.07 ± 0.79

* Dry weight basis; Values are mean of five observations and are significant at $p<0.0001$

Fenugreek is a fast growing diosgenin-containing plant and has an added advantage when compared to the slow-growing *Dioscorea* species; though the latter contains a higher concentration of diosgenin in their tubers (Savikin-Fodulovic et al., 1998).

Other sapogenins (which include smilagenin, neotigogenin, yuccagenin, neogitogenin) content in the crude extract is 12.68 µg/mg and in purified extract is

34.07 μg/mg (Table 3.2). The total sapogenins calculated in the crude extract is 54.74 μg/mg and in purified extract is 215.02 μg/mg.

The effectiveness of the purification procedure is demonstrated in Table 3.3. The purification resulted in more than four fold increase in the diosgenin content. In addition, several non-sapogenin peaks were present in crude extract (retention time 5-10 min), that were not observed in the purified extract (Figures 3.3 and 3.4). The simplicity of the purification procedure and its effectiveness may make it a useful procedure for the steroid industry.

Table 3.3. Purification of diosgenin (in percent) by the sep-pak method

	% of diosgenin (N)	
Analysis	Extract	Purified Extract
1	2.43 (3)	11.19 (3)
2	2.65 (3)	10.65 (3)
3	2.78 (3)	12.45 (2)
Mean of three analysis	2.62 ± 0.10*	11.43 ± 0.53*

*$p<0.05$

3.3.4. Determination of sugars in crude extract and purified extract

The gas chromatographic analysis revealed the presence of different sugars in the crude extract and purified extract. The sugars present in both the sources are rhamnose, fucose, arabinose, xylose, mannose, glucose and galactose (Figures 3.5 and 3.6 respectively). Glucose is the main sugar in both crude and purified extract. The

second major sugar present in both crude extract and purified extract is rhamnose. The crude extract contains some amount of mannose and galactose. Whereas purified extract contains negligible amount of mannose (Table 3.4). The small amount of fucose and arabinose was determined in both crude extract and purified extract. The sources of these two sugars are not clear.

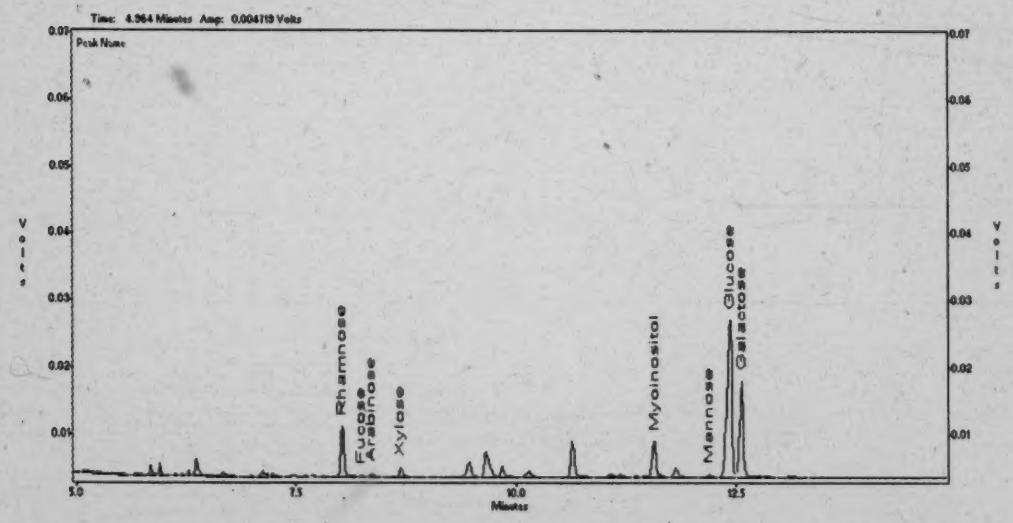

Figure 3.5. GC chromatogram showing sugars present in crude extract

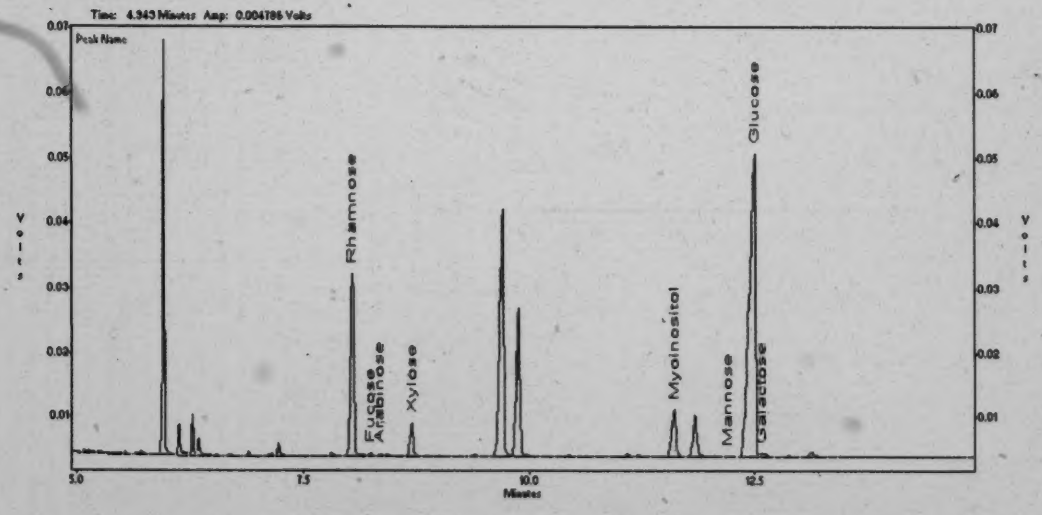

Figure 3.6. GC chromatogram showing sugars present in purified extract

Table 3.4. Estimation of sugars (%) present in extract and purified extract

Carbohydrate (%)	Extract	Purified Extract
Rhamnose	3.91* ± 0.23	10.51* ± 0.27
Fucose	0.10 ± 0.05	0.11 ± 0.01
Arabinose	0.14 ± 0.03	0.07 ± 0.02
Xylose	0.44* ± 0.02	1.18* ± 0.08
Mannose	0.13 ± 0.04	0.02 ± 0.00
Glucose	10.64* ± 0.03	21.00* ± 0.32
Galactose	5.15* ± 0.134	0.26* ± 0.02

*Mean values and are significant at $p < 0.05$.

There is no significant effect of duration for hydrolysis (tested in the experiment) on the sugar extraction of the extract and purified extract. The total sugar concentration was highest (20.2%) with 2.5 hr of hydrolysis of extract. Whereas, the 1 hr of hydrolysis is sufficient for yielding highest amount (33.06%) of sugars from the purified extract (Figure 3.7).

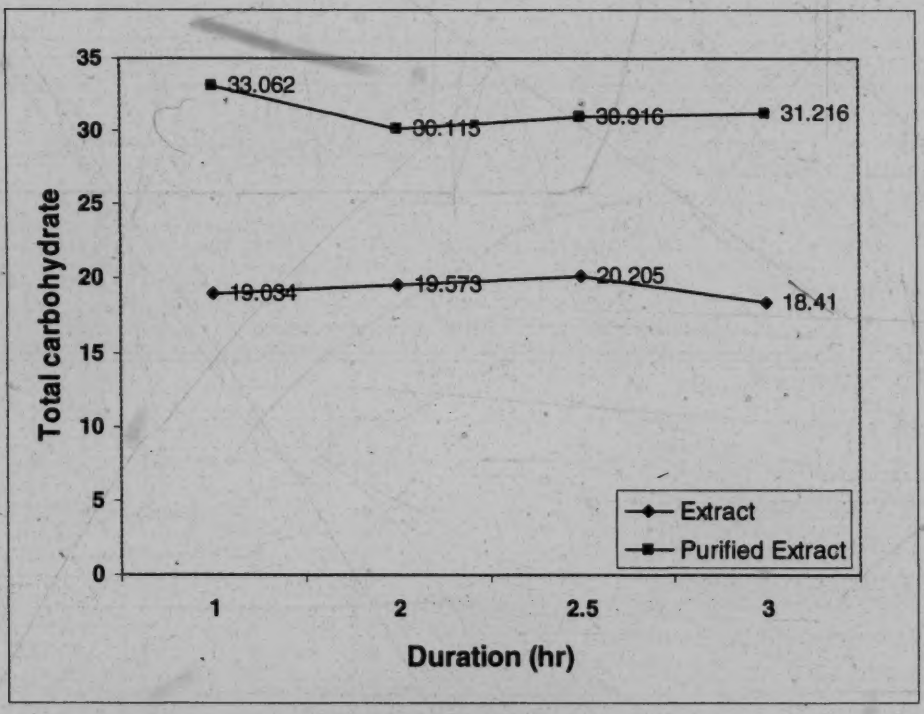

Figure 3.7. Effect of duration of hydrolysis on total sugars (%) extraction of crude extract and purified extract.

The acid hydrolysis of the fractionated portions of methanolic extract of the fenugreek seeds showed the presence of D-glucose, L-rhamnose, D-xylose (Hardman et al., 1980; Gupta et al., 1984; 1985; 1986). The glycoside isolated by Hardman et al. (1980) had a structure, (25S)-22-O-methyl-5α- fyrostan-3β, 22,26-triol 3-O-α-rhamnopyranosyl (1→2)[-β-D-glucopyranosyl (1→3)]-β-D-glucopyranoside-26-O-β-D-glucopyranoside; so in this glycoside glucose and rhamnose are attached to sapogenin.

Gupta et al. (1984) determined the structure of two glycosides in fenugreek seed extract. On hydrolysis with 2N HCl, one glycoside gave D-glucose and L-rhamnose and the second one gave D-glucose, D-xylose and L-rhamnose.

The structure elucidation of other glycosides of fenugreek demonstrated the presence of D-glucose and L-rhamnose (Gupta et al., 1985, 1986). The results of the present study are also similar to the findings of previous researchers. So, it could be concluded that the fenugreek of the variety 'Amber' possesses the saponins, which contain glucose, rhamnose and xylose attached to its sapogenin part. It is not clear how and how many sugar molecules are attached to the sapogenin. Further study is required for structural elucidation of these saponins present in the crude extract and purified extract of this fenugreek variety 'Amber'.

3.4. References

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CHAPTER 4

Cholesterol and bile acid binding capacity of crude extract and purified extract:

4.1. Introduction

Some of the saponin containing plants, which have been used in food and beverage products, is listed in the Table 4.1.

Table 4.1. Significant food and beverage plants which contain saponins

Latin Binomial	Common name	Plant parts used
Allium cepa L.	Onions	Bulbs
Allium sativum L.	Garlic	Cloves
Camellia sinensis L.	Tea	Leaves
Lens culinaris L.	Lentils	Seeds
Panax quinquefolium L.	Ginseng	Roots
Phaseolus mundo L.	Black gram/Mung beans	Seeds
Pisum sativum L.	Green beans	Seeds
Trigonella foenum-graeccum L.	Fenugreek	Seeds, Leaves
Quillaja saponaria	Quillaja	Bark

Isolated saponins and foods containing saponins have been shown to lower plasma cholesterol concentrations in a number of animal species (Malinow et al., 1977a; Oakenfull et al., 1979) and it has been suggested that foods containing saponins could be important to formulate hypocholesterolaemic diets for human consumption (Potter et al., 1980; Malinow et al., 1981). In recent years, the mechanism by which saponin lowers cholesterol is creating considerable interest among the researchers. Feed containing triterpene glycosides given to hens reduce the amount of cholesterol both in blood and tissues (Cheeke, 1971).

Saponins fed to rats reduce plasma triglyceride and cholesterol content, by up to 30% and 25%, respectively (Lutomski, 1983). Lipid concentration in liver has been found to be reduced by consuming alfalfa saponin in mice (Reshef et al., 1976). Soybean saponins reduce the intestinal uptake of cholesterol in rats (Sidhu et al., 1987). Total cholesterol and triglyceride have been reduced by ginseng extract without effecting HDL cholesterol in rabbits (Moon et al., 1984); ginseng saponin, moreover reduce the spread of arteriosclerosis in a rabbit model (Koo, 1983). The elevation of blood cholesterol level in rats has been reported to be prevented by adding fenugreek seeds to a hypercholesterolaemia induced diet (Sharma, 1984). A crude saponin fraction isolated from fenugreek reduced serum cholesterol in rats (Sharma, 1986). Chronic oral administrations of fenugreek extract have been demonstrated to increase food intake and motivation to eat in rats. An increase in plasma insulin and a decrease in total cholesterol and VLDL, LDL have also been observed (Petit et al., 1993). An ethanolic extract of fenugreek seeds, which contain a

hypocholesterolaemic component, has also been shown to reduce plasma cholesterol levels in rats (Stark and Madar, 1993).

The mechanism of action by which saponin lowers cholesterol level, however, is yet to be determined. There has been a suggestion that saponins remain within the gastrointestinal tract unabsorbed (Birk, 1969) and that they bind cholesterol, producing an insoluble complex and thereby cholesterol absorption is prevented (Malinow et al., 1977b). Saponins are believed to bind bile acids and increase faecal excretion of bile acids (Oakenful et al., 1979, 1984). Bhat et al. (1985) reported that an oral administration of fenugreek to rats increased bile acid synthesis in the liver. This was attributed to the increased breakdown of cholesterol to bile acid.

The aim of the work presented in this chapter was to find out whether the crude extract and purified extract have an ability to bind cholesterol and bile acid *in vitro*.

4.2. Materials and Methods

4.2.1. *In vitro* cholesterol binding

4.2.1.1. Preparation of standard curve

A) Standard cholesterol solution (1 mg/ml) was prepared by dissolving 100 mg of pure, dry, ash-free cholesterol (Sigma Chemical Co, St Louis, MO, USA) in 100 ml of glacial acetic acid.

B) Ferric chloride solution was prepared by dissolving 10 g of ferric chloride (Fisher Scientific, Nepean, ON, Canada) in 100 ml of glacial acetic acid.

C) Color reagent was prepared by diluting 2 ml of ferric chloride solution with 200 ml of analytical grade sulfuric acid (Fisher Scientific, Nepean, ON, Canada).

Different concentrations of test solutions were prepared by mixing 0.1, 0.2 and 0.3 ml of standard cholesterol solution with 3 ml of glacial acetic acid into clean dry 30 ml test tubes. Distilled water (0.1 ml) was added to each tube and mixed thoroughly. A blank was prepared by mixing 3 ml of glacial acetic acid and 0.1 ml of distilled water. Color reagent (2 ml) was added carefully allowing it to flow down the side of the test tube, thus producing two layers. A light brown color first appeared, which turned into intense purple within a minute. When the tube has cooled to room temperature the absorbance was measured in a spectrophotometer at 560 nm using a 1 cm cuvette. The standard curve was obtained by plotting absorbance against concentration of cholesterol.

4.2.1.2. Cholesterol binding with crude extract and purified extract

The cholesterol binding capability of crude extract and purified extract was estimated according to the method described by Cayen and Dvornik (1979). The cholesterol solution (1 mg/ml) was prepared by dissolving cholesterol in ethanol-ether

(1:1, v/v). Solutions (4 mg/ml) of crude extract and purified extract were prepared by dissolving the extracts (crude and purified separately) in ethanol-ether (1:1, v/v). In three different test tubes (screw cap), 3 ml each of the cholesterol solutions were mixed with 3 ml of each extract solution, capped and incubated at room temperature overnight. A control has also been set and incubated under similar conditions. After centrifugation, the cholesterol content with or without the extracts was measured calorimetrically according to Zlatkis et al. (1953). The difference in cholesterol content with or without the extract indicated the amount of precipitated cholesterol.

4.2.2. *In vitro* bile acid binding

Sodium taurocholate and radioactive sodium taurocholate (specific activity: 57 m*Ci*/mMol) were obtained from MP Biochemicals Inc., Aurora, OH and Amersham Biosciences, Little Chalfont, Buckinghamshire, UK respectively. Mono- and di-basic potassium phosphate (K_2HPO_4 and KH_2PO_4) were purchased from BDH Inc, Toronto, ON. Microcon centrifugal filter devices (3000 molecular weight cut off point) of 0.5 ml capacity were procured from Millipore Corporation, Bedford, MA.

Different concentrations (1, 2, 4, 8 mg/ml) of crude extract and purified extract solutions were prepared by dissolving them in water (Fisher Scientific, Nepean, ON, Canada). Labeled bile acid solution (20 µmol/ml) was prepared by dissolving sodium taurocholate in phosphate buffer (20 µM, pH 6.5). In separate tubes, 500 µl of the labeled bile acids were mixed with 500 µl of crude extract and

purified extract. The mixtures were then incubated at 37°C for 1 hr while shaking in a water bath. At the end of the incubation period, 250 µl of the incubation mixture was transferred into a centrifugal filter device and centrifuged at 30,000xg for 20 min. An aliquot of 50 µl of the filtrate was taken in scintillation vial and 12 ml of the scintillation fluid (Fisher Scientific, Nepean, ON, Canada) was added and counted in a liquid scintillation counter (Beckmann Coulter Canada Inc., Mississauga, ON). A control was also run maintaining the same experimental conditions without adding binding agent (crude extract and purified extract). The disintegrations per minute (DPM) counts are considered as the counts for the free bile acids.

4.2.3. Effect of purified extract on bile acid binding

Different concentrations of bile acid were tested against different concentration gradient of saponins. The experimental protocol was similar to that described in section 4.1.2. A stock solution (30 µmol) of bile acid was prepared by dissolving sodium taurocholate in phosphate buffer (pH 6.5). Radioactive taurocholate was added to the stock solution. Working solutions were prepared from the stock solution at concentrations of 2.5, 5.0, 10, 20 and 30 µmol by adding appropriate amounts of buffer solution.

A stock solution (5 mg/ml) was prepared by dissolving purified extract in phosphate buffer. Working solutions of 1.0, 2.0, 3.0, 4.0 and 5.0 mg/ml were prepared

by adding appropriate amounts of buffer solution to the stock solution of the purified extract.

Equal volume of labeled taurocholate solutions were added to purified extract solution to test different combinations of bile acid and purified extract. The final experimental solutions for bile acid were 1.25, 2.5, 5.0, 10 and 15 μmol; similarly, those for purified extract were 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml. The mixture was incubated maintaining similar conditions as described earlier. The unbound taurocholate was measured as mentioned in section 4.1.2.

4.2.4. Statistical analysis

Data obtained were subjected to analysis of variance using general linear model procedure in SAS (Statistical Analysis System, Version 7.0, SAS Institute Inc., Cary, NC, USA). Means of different treatments were compared using Tukey's studentized range test in SAS.

4.3. Result and Discussion

4.3.1. Cholesterol binding

The solution of extract added to the solution of cholesterol developed precipitation after standing overnight at room temperature. Under the same experimental conditions, a solution of purified extract precipitated less amount of

cholesterol. The amount of free cholesterol was calculated from the absorbance data and using the standard curve (Figure 4.1).

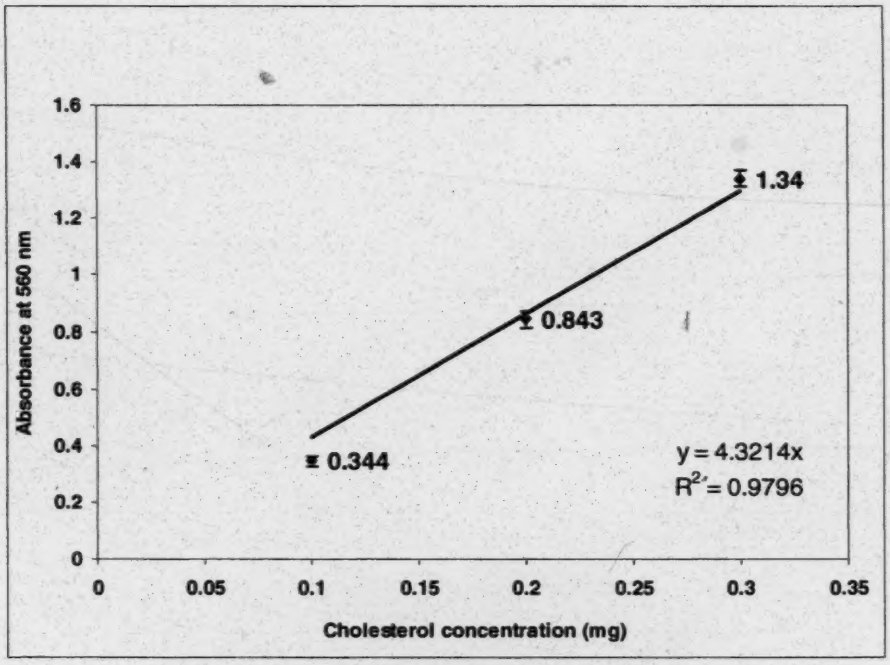

Figure 4.1. Standard curve for cholesterol

Under the same experimental conditions purified extract precipitated 35.38% of cholesterol whereas crude extract precipitated 41.83% (Figure 4.2), the difference being statistically significant ($p < 0.001$). It has been found that the crude extract has more capacity of *in vitro* binding of cholesterol than the purified extract.

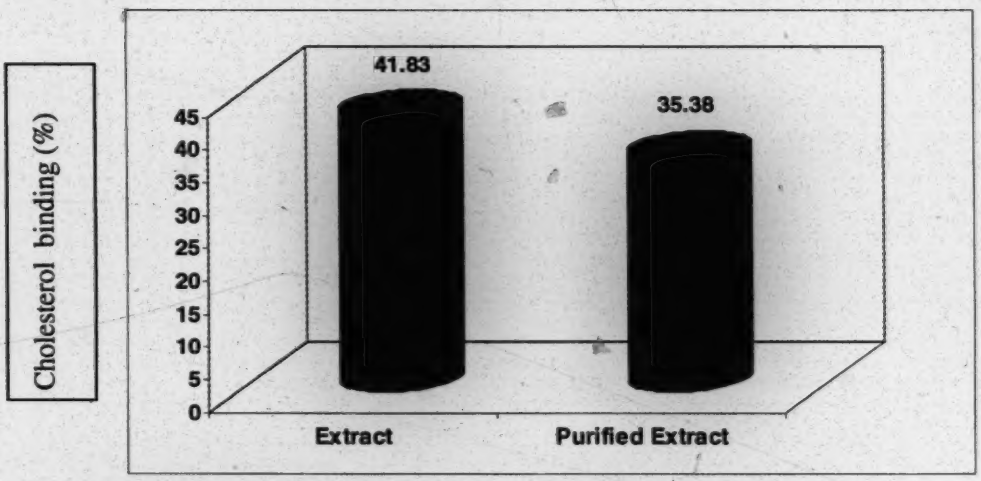

Figure 4.2. Effect of crude extract and purified extract on cholesterol binding; Values are means of nine observations and are significantly different, $p<0.001$

Some saponins interact directly with cholesterol producing an insoluble complex which prevents cholesterol absorption in small intestine (Malinow et al., 1977b). It seems that the crude extract which is a mixture of saponin, fiber and different pigments (e.g. anthocyanins) has better capability in binding cholesterol. However, the purified extract, which is a more concentrated form of saponins are less capable of binding cholesterol. A detailed animal study is required to find out how the crude extract and purified extracts perform under controlled physiological conditions.

4.3.2. Bile acid binding

Both the crude extract and the purified extract showed an appreciable amount of binding capacity with labeled [^{14}C] sodium taurocholate. The purified extract

displayed a grater binding capacity than the crude extract, irrespective of the concentration of taurocholate used. The bile acid binding capacity was found to increase linearly with the concentration of either the crude extract or the purified extract (Figures 4.3 and 4.4 respectively).

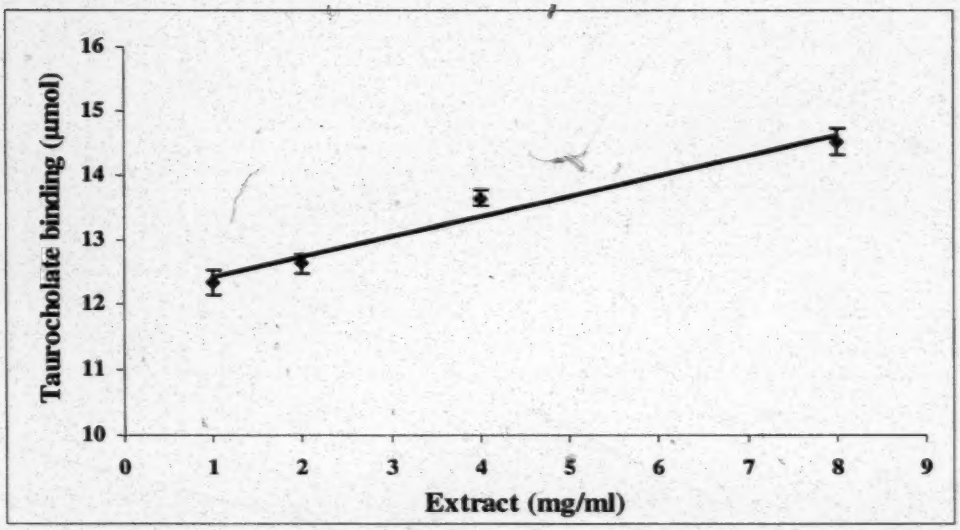

Figure 4.3. The dose dependent binding of [14C] taurocholate by crude extract

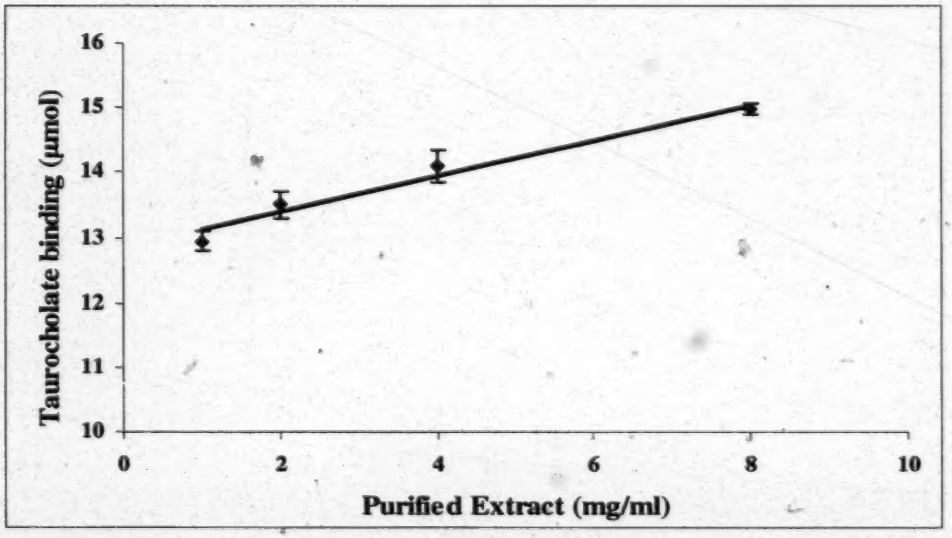

Figure 4.4. The dose dependent binding of [14C] taurocholate by purified extract

When increased concentrations of bile acids were incubated for binding with a fixed amount of purified extract; the results clearly demonstrated that the purified extract could bind higher amounts of bile acid when higher amounts of bile acid was available in the media. Results were similar for different concentrations of purified extract tested in the experiment (Figure 4.5).

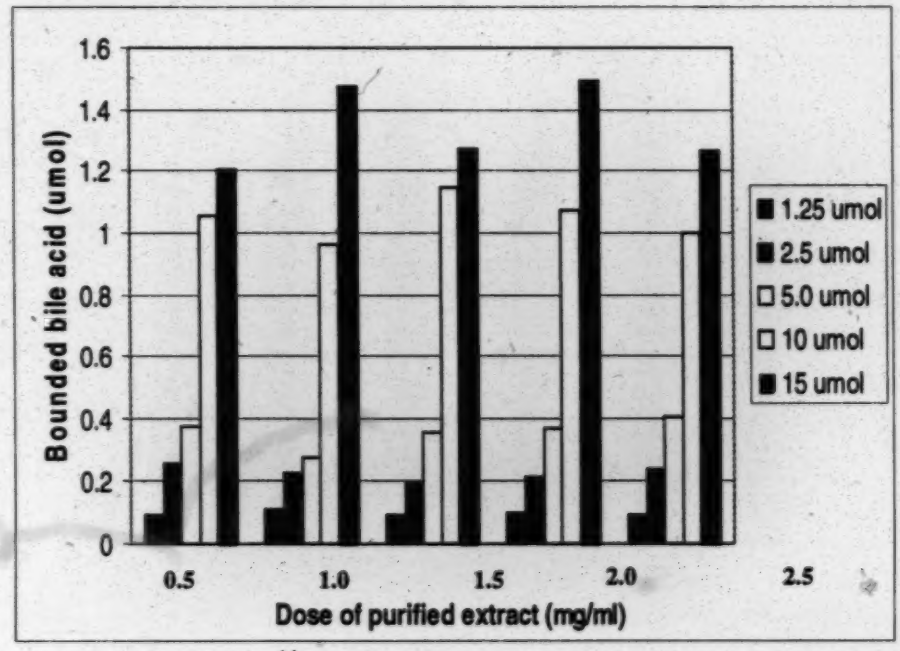

Figure 4.5. Bile acid ([^{14}C] sodium taurocholate) binding by purified extract

Saponin interacts with bile acids and increased faecal excretion of bile acid has been observed in response to feeding saponins (Oakenful et al., 1979, 1984). It has been shown by Oakenful and Sidhu (1983) that a commercial purified saponin from soapwort (*Saponaria officinalis*) formed large mixed micelles with bile acid and that the absorption of bile acid from the micellar solutions was greatly reduced. The results of this study also suggest that the saponin has an ability to bind bile acids in a

dose dependent manner. Thus, there may be a simple physico-chemical explanation for the effect of fenugreek saponin on cholesterol and bile acid metabolism.

A further study is required to find how and at what concentration the crude extract and purified extract could bind bile acid in a physiological set up. Then combining the results of these studies, will allow us to understand how and at what dose the crude extract and purified extract containing saponin will control bile acid and cholesterol metabolism and thus help in controlling hypercholesterolaemia.

4.4. References

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CHAPTER 5

General Discussion

Saponins, a heterogeneous group of amphiphilic compounds are of different kinds. Most saponins are haemolytic, can bind cholesterol, and form foams (Price et al., 1987). Studies reported so far on the effects of saponins on cholesterol homeostasis concern mainly the triterpenoid saponins from lucrene (Malinow, 1984) and steroidal saponin from soyabean (Sidhu et al., 1987) which reduce intestinal uptake of cholesterol. It has also been reported that steroidal saponin prevents or lowers cholesterol in monkeys (Malinow et al., 1978). Saponins derived from lucerne (*Medicago sativa*) were found to reduce plasma cholesterol levels by direct binding of dietary saponins with cholesterol in the digestive tract and subsequent excretion in the feces (Malinow et al., 1977, 1981; Story et al., 1984). Other types of saponins affect cholesterol metabolism indirectly by interfering with bile acids and increasing their faecal excretion (Oakenfull et al., 1984).

Fenugreek seed saponins are steroidal in nature with diosgenin as the main sapogenin (Mahato et al., 1982). Diosgenin has various effects on cholesterol metabolism, one of the most important being the capacity to lower plasma cholesterol concentration in chickens and rabbits fed cholesterol (Laguna et al., 1962). This hypocholesterolaemic effect has been suggested to be dependent on the capacity of diosgenin to inhibit cholesterol absorption, to decrease liver cholesterol concentration, to increase biliary cholesterol secretion and increase faecal excretion of neutral sterols (Cayen and Dvornik, 1979; Uchida et al., 1984; Ulloa and Nervi, 1985). Furthermore,

Malinow (1985) has shown that diosgenin glucoside was more efficient than diosgenin in reducing intestinal absorption of cholesterol. At comparable small doses, diosgenin glucoside inhibited cholesterol absorption *in vivo* and *in vitro*, whereas diosgenin did not (Malinow, 1985; Malinow et al., 1987). Sauvaire et al. (1991) have examined the transformation of fenugreek subfractions rich in steroid saponins during their passage through the digestive tract, to determine the relative contribution of saponins and/or diosgenin and other steroid sapogenins to the hypocholesterolaemic effect of fenugreek seeds. In this study (Sauvaire et al., 1991) faecal samples from alloxan diabetic dogs fed the fenugreek subfractions were analysed by capillary gas chromatography/mass spectrometry for the presence of sapogenins. Their results suggest that saponins, are in part (about 57%), hydrolysed into sapogenins (diosgenin, smilagenin, gitogenin) in the digestive tract. The location of fenugreek saponin hydrolysis in the digestive tract was not determined. The authors concluded that saponin hydrolysis does occur, presumably in the stomach and/or in the proximal small intestine (Sauvaire et al., 1991). Since hydrolysis was incomplete, saponins may be implicated, alone or together with sapogenin, in the observed hypocholesterolaemic effect of fenugreek seeds.

Another possible mechanism for the inhibition of bile salt absorption may be primarily mechanical, due to the formation of a physical barrier by fenugreek extracts, such as the gel fraction. The study by Ribes et al. (1987) showed that a fibre-rich subfraction ('a') separated from the saponins also displayed a hypocholesterolaemic effect. Galactomannan derived from fenugreek seeds has been reported to inhibit intestinal bile acid absorption, reducing the efficiency of their

enterohepatic circulation and subsequently decreasing plasma cholesterol level (Madar and Shomer, 1990).

All of the above mentioned studies used the whole or defatted fenugreek seeds for understanding its activity of hypercholesterolemia. As a matter of fact fenugreek seeds are also a rich source of fiber (galactomannan) along with saponins. The desaponified fraction of fenugreek seed produced a significant hypocholesterolaemic effect (Sharma et al., 1990; Marles et al., 1995; Zia et al., 2001). Moreover, all the effects of fenugreek on human health have been identified using the Asian fenugreek and no such data are available to date using the fenugreek of Canadian origin.

Therefore, the purpose of this present study was to investigate the effect of Canadian fenugreek seed extract rich in saponin on cholesterol and bile acid binding to understand the possible mechanisms for lowering blood cholesterol. This was accomplished by developing a suitable procedure for the saponin extraction and purification; analyzing the crude extract and purified extract and finally determining the cholesterol and bile acid binding capacities of these extracts.

An important aspect of the modern use of plant extracts as pharmaceutical preparations is the characterization and determination of the individual active constituents. Such is also the case for saponin preparations, which often require sophisticated techniques for their isolation, structure elucidation and the analysis of their components. When the biological testing of pure compounds is to be performed, it is necessary to isolate them in sufficient quantity and purity. Therefore, the isolation and characterization of the fenugreek saponins is vital in order to investigate their biological activities.

The extraction procedures have to be as mild as possible because certain saponins can undergo the following transformations: a) enzymatic hydrolysis during water extraction (Domon and Hostettmann, 1984; Kawamura et al., 1988), b) esterification of acidic saponins during alcohol treatment, c) hydrolysis of labile ester group and d) transacylation.

Keeping all this information in hand, extra care was always taken throughout the extraction and purification of the fenugreek seed extracts and individual steps were monitored using TLC. Upon reviewing the literature, it has been found that ethanol is a better solvent than methanol for saponin extraction from fenugreek seeds; hence, ethanol has been chosen for the extraction of saponin from seeds. In the present study, ethanol (80%) has been found to be the best solvent for the extraction of saponin from defatted fenugreek seeds at a temperture of 60°C and with a constant stirring for 4 h.

The isolation of the pure saponins requires one or more chromatographic separation steps in order to remove other polar constituents of alcoholic or aqueous plant extract. A variety of modern separation techniques (Hostettmann et al., 1986, 1991; Marston and Hostettmann, 1991b) such as flash chromatography, low pressure liquid chromatography (LPLC), medium pressure liquid chromatography (MPLC) and HPLC are available, but a large number of separations (especially the preliminary fractionation work) reported in the literature are still carried out by conventional open column chromatography. The best result is usually achieved by strategies, which employ a combination of methods.

Open-column chromatography is often used as a first fractionation step for a crude saponin mixture but in certain cases may yield pure products (Hostettmann and Marston, 1995). Silica gel chromatography with chloroform-methanol-water as solvent is the most popular method and is still used in majority of separations.

As an addition to normal silica gel, coarse RP (reverse phase) adsorbents are employed in open-column chromatography of saponins. In order to remove interfering materials from a saponin preparation, a clean up on Sep-Pak C_{18} (Wagner et al., 1985; Wagner and Reger, 1986a, b; Pietta et al., 1986; Ireland and Dziedzic, 1986b; Bushway et al., 1986; Guedon et al., 1989) is reported. For glycoalkaloids, sample preparation with Sep-Pak C_{18} and Sep-Pak NH_2 cartridges has been reported (Saito et al., 1990a). The C_{18} Sep-Pak cartridge has been found as a suitable solid support for the purification of saponin. In this study, by using C_{18} Sep-Pak cartridge for the purification of the fenugreek extract, a five fold increase in diosgenin content has been achieved. This one step procedure could be very much useful to the industry for the purification of the fenugreek saponin. Further analysis of the crude extract and purified extract showed that purification improves the diosgenin concentration to 11% in comparison to the 2.27% diosgenin present in the crude extract.

Different methods have been employed by the different researcher for the qualitative and quantitative determination of saponins: haemolysis, piscicidal activity, gravimetry, spectrophotometry, TLC, GC, and HPLC.

The qualitative analysis of saponins by TLC is of great importance for all aspects of saponin investigations. Pure saponin as well as crude extract both could be analyzed by TLC plates (usually silica gel) which are inexpensive, rapid to use and

require no specialized equipment. A number of visualization reagents are available for spraying onto the plates.

The most frequently used solvent for TLC is chloroform-methanol-water (65: 35:10 v:v:v) (Kawasaki and Miyahara, 1963). Similar solvents have been used for qualitative determination of fenugreek saponins in the crude extract and purified extract. A characteristic color (brick red) developed by spraying Erlich's reagent onto the developed silica gel TLC plate and proved that the saponins present in fenugreek is of furostanol type.

In this study, further characterization of the crude extract and purified extract was done using different techniques. It has been found that the extract contains significant amount of soluble dietary fiber (SDF). Due to the presence of SDF as well as saponins, using the crude extract may be more beneficial than the purified extract in relation to the health benefits.

Saponins are glycosides in which the hemiacetal hydroxyl groups of saccharides in their cyclic pyranose or furanose forms build acetals with triterpene or steroid residue. The ether linkage between the hemiacetyl hydroxyl and the triterpene or steroid is known as a glycosidic linkage. The monosaccharide constituents of the oligosaccharides are also bound by ether linkages (interglycosidic bonds).

Upon complete hydrolysis of the glycoside, the glycoside linkage is cleaved to liberate the component monosaccharides and the non-carbohydrate moiety. Numerous chemical reactions and methods have been employed for breaking down saponins into smaller units for analysis. The most common one is acidic hydrolysis by refluxing the saponin in acid for a fixed length of time. The aqueous solution remaining after

hydrolysis is extracted with diethyl ether, chloroform or ethyl acetate to obtain the aglycone. Extraction of sugars from the aqueous layer is performed with pyridine, after neutralizing the solution with alkali.

In the present study, the crude extract and purified extract were hydrolyzed by refluxing propanol-sulfuric acid mixture; their sapogenins were determined using a GC technique and it has been found that the fenugreek contains a number of saponins. This finding is supported by numerous studies, which reported that the fenugreek saponins are a source of diosgenin, yamogenin, neotigogenin, yuccagenin, gitogenin and neogitogenin (Puri et al., 1976; Taylor et al., 2000). Due to the presence of diosgenin (major sapogenin present in fenugreek seed) fenugreek could be considered as an alternative source of diosgenin, which is a very important constituent of steroids industry.

The comparative study of the chromatograms of crude extract and purified extract of fenugreek seed revealed that the probable sugars present as glycone moiety are rhamnose, glucose and xylose. This observation is similar to those of Hardmann et al., (1980); Gupta et al., (1984, 1985, 1986). However, this experimental data from the present study can not suggest how sapogenins are attached with sugar molecules, at the same time it could not suggest us the type of saponins in fenugreek seed of Canadian origin i.e. monodesmosidic or bidesmosidic.

Cholesterol is one of the most highly recognized molecules in human biology, in part because of a direct relationship between its concentrations in blood and tissues and the development of atherosclerotic vascular disease. Cholesterol, which is transported in the blood in lipoproteins because of its absolute insolubility in water,

serves as a stabilizing component of cell membranes and as a precursor of the bile salts and steroid hormones. As a major component of blood lipoproteins, cholesterol can appear in its free, unesterified form in the outer shell of these macromolecules and as cholesterol esters in the lipoprotein core.

Cholesterol is obtained from the diet or synthesized by a pathway that occurs in most cells of the body, but to a greater extent in cells of the liver and intestine. The precursor of cholesterol synthesis is acetyl CoA, which can be produced from glucose, fatty acids or amino acids. HMG-CoA reductase is the major rate limiting enzyme in the cholesterol biosynthesis pathway.

Cholesterol absorption by the intestinal cells is a key regulatory point in human sterol metabolism because it ultimately determines what percentage of the 1,000 mg of the biliary cholesterol produced by the liver each day and what percentage of the 300 mg of dietary cholesterol entering in the gut per day is eventually absorbed into the blood. In normal subjects, approximately 55% of this intestinal pool enters the blood through the enterocyte each day.

The transportation of the cholesterol is mediated by the lipoproteins and the chylomicrons. Cholesterol is packaged in chylomicrons in the intestine and in very-low-density lipoprotein (VLDL) in the liver. As the triacylglycerols of the blood lipoproteins are digested by lipoprotein lipase, chylomicrons are converted to chylomicron remnants, and VLDL is converted to intermediate-density lipoprotein (IDL) and subsequently to low-density lipoprotein (LDL). These products return to the liver, where they bind to receptors in the membranes and are taken up by the endocytosis and digested by the lysosomal enzymes. LDL is also endocytosed by the

nonhepatic (peripheral) tissues. Cholesterol and other products of the lysosomal digestion are released into the cellular pools. The liver uses this recycled cholesterol, and the cholesterol that is synthesized from the acetyl CoA, to produce VLDL and to synthesize bile salts.

The above mentioned homeostasis may break under certain physiological and/or disease condition. Under such condition the elevation of the total cholesterol and specially LDL-c is a common phenomenon in human physiology. Elevated levels of the cholesterol in the blood are associated with the formation of the atherosclerotic plaques that occlude blood vessels, causing heart attacks and strokes. Although high levels of the LDL cholesterol are especially atherogenic, high levels of HDL cholesterol are protective because HDL particles are involved in the process of removing cholesterol from tissues, such as from lining cells of vessels and returning to the liver. The excretion of the cholesterol from the body can only be mediated by the bile acid formation and their subsequent formation into secondary bile salt. However, greater than 95% of the bile salts are reabsorbed in the ileum and return to the liver via enterohepatic circulation (via portal vein).

Although the fecal excretion of the bile salts is relatively low, it is a major means by which the body disposes of the steroid nucleus of cholesterol. Because the ring structure of the cholesterol cannot be degraded in the body; it is excreted mainly in the bile as free cholesterol and bile salts.

Some of the experimental studies revealed that fenugreek seeds have the ability to lower cholesterol by binding bile acid. Sharma (1984) suggested that fenugreek seeds, added to a hypercholesterolaemia-inducing diet in rats, prevented

elevation of plasma cholesterol levels by increasing faecal bile acid and cholesterol excretion.

Stark and Madar (1993), using an ethanol extract from ground fenugreek seeds, found that the saponin-rich isolate lowered plasma cholesterol levels in hypocholesterolaemic rats by 18-26%. The ethanol extract inhibited bile acid absorption in inverted segments of rat intestine in a dose dependent manner.

In the present study, crude extract and purified extract of Canadian fenugreek seed were tested for their cholesterol and bile acid binding capacity in an *in vitro* experiment. In two separate experiments, cholesterol and bile acid binding capacity of the crude extract and purified extract were measured. The result showed that crude extract and purified extract of Canadian fenugreek can bind cholesterol and bile acid in a dose dependent manner. By binding cholesterol and bile acid the availability of both these compounds could be limited for absorption in the intestine and their subsequent excretion in feaces may be an effective means for lowering cholesterol pool in the body.

The first limitation of the study, however, is the unavailability of the saponin standards. For this reason to determine the saponin content, indirect methods have always been used by different researchers as well as in this study. The loss of the active compounds throughout the time of processing cannot be estimated and the concentration of the compounds is likely underestimated compared to actual amount in the sample.

The second limitation of the study is in the determination of the free cholesterol and bile acid. Filters with 3000 dalton as a cut off were used. Thus any

bound bile acids which are less than 3000 dalton that could have through pass the filter, and be measured as an unbound portion. The estimation of the bound portion may therefore be less than the actual binding. Further studies are required to overcome this problem.

5.1. Conclusion

The present study demonstrated that the ethanolic extract of defatted fenugreek seeds of Canadian origin is a rich source of saponin. The extract can be purified with simple open column chromatography procedure using C18 as a solid support and methanol as an eluting solvent.

The crude extract and purified extract both have the capacity to bind cholesterol and bile acid under an experimental condition, which in turn may be beneficial for lowering cholesterol.

Further research is required for the complete characterization of the saponins in the Canadian fenugreek. Of all the modern methods for the structure elucidation of glycosides, NMR (Nuclear Magnetic Resonance) spectroscopy provides the most complete information, with or without prior structural knowledge (Agarwal, 1992). It is the only approach which can, in principle, give a complete structure of saponins without resorting to any other method. Thus, it is recommended to use NMR for complete elucidation of the structures of saponin present in Canadian fenugreek seeds.

The crude extract and purified extract of fenugreek were both found to be competent enough to bind bile acid and cholesterol. However, how these two products will work in physiological condition is not known. An elaborate animal study is required to determine how effective they are in exerting this binding effect in an *in vivo* condition. On the basis of the results of animal studies the efficacy of the product in human subjects should be determined for drawing a final conclusion on the effectiveness of the saponins of Canadian fenugreek in lowering cholesterol.

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